Parturition: Laboratory and Clinical Investigations

Janet Elizabeth Brennand MB ChB, MRCOG

Thesis submitted for the degree of
Doctor of Medicine
University of Edinburgh
1998

Declaration

This thesis has been composed by myself, and I have been responsible for recruitment of patients, clinical management and laboratory studies unless otherwise acknowledged.

The contents of this thesis have not been submitted elsewhere for any other degree, diploma, or professional qualification.

Janet E Brennand

Contents

Acknowledgements

I am extremely grateful to Ian Greer for his continued support and advice throughout the compilation of this thesis.

Particular thanks are extended to Rose Leask for her patience, skill and expertise in assisting with, and supervising the laboratory work, and for training me in the techniques of explant and cell culture.

I thank Rodney Kelly for his valuable advice in techniques of radioimmunoassay, and for providing the antibodies for the laboratory experiments.

I thank Catherine Elliott who recruited nine of the patients for the mifepristone trial, and Craig Leitch and Min Min Chou for recruiting patients at their respective centres for the relaxin trial. I also acknowledge the support of Hoechst Marion Rousell and Genentech in these clinical studies.

I gratefully acknowledge the Consultant staff of Simpson Memorial Maternity Pavilion, Edinburgh who allowed their patients to be recruited for the clinical studies, and the patients themselves who were willing participants in these trials.

Finally, I am indebted to Andrew Calder for the opportunity to perform this research, and for his encouragement and guidance not only with this thesis but throughout my career.

Parturition: Laboratory and Clinical Investigations.

The importance of the appropriate timing of parturition is highlighted by the fact that preterm labour accounts for 85% of the deaths of normally formed babies. In addition, there are clinical situations where induction of labour is required. Attempts to reduce the incidence of preterm labour and facilitate induction require an understanding of the normal processes of labour at term.

In this thesis the physiology of parturition is considered, with emphasis on prostaglandin biosynthesis and metabolism, and the regulation thereof, processes which are thought to be pivotal in the mechanism of labour. The physical and biochemical changes associated with cervical ripening are discussed, as are mediators of this process. Finally, the current understanding of ovine parturition and potential similarities with humans is reviewed.

The laboratory studies described employ the techniques of explant and cell culture of fetal membranes, namely amnion and chorion, to investigate firstly the changes in synthesis and metabolism of prostaglandins E_2 and $F_{2\alpha}$ (PGE_2, $PGF_{2\alpha}$) that occur within these tissues in association with labour. Prostaglandin production by amnion is increased without any effect on metabolism by chorion. The effect of amniotic fluid on prostaglandin synthesis and metabolism in cell culture was then explored, and, having demonstrated that amniotic fluid from labouring women stimulates PGE_2 and $PGF_{2\alpha}$ production, the mechanisms controlling the response to amniotic fluid were investigated, and found to be dependent on new protein synthesis and protein kinase C.

In the clinical situation pharmacological ripening of the cervix was studied in two randomised, placebo-controlled clinical trials. The efficacy of recombinant human relaxin and mifepristone (RU 486) was investigated in women with an unfavourable cervix, modified Bishop score ≤ 4, in whom induction of labour was indicated. Vaginal administration of relaxin (1, 2 and 4 mg) was without effect on cervical ripening. The first part of a dose finding study with mifepristone demonstrated that a dose of 50 mg may have some preparatory effect on the cervix with regard to ripening, but that a higher dose is required for successful induction of labour.

Publications

The following papers have been published based on the text of this thesis:

Brennand J.E, Leask R, Kelly R.W, Greer I.A and Calder A.A. (1995) Changes in prostaglandin synthesis and metabolism associated with labour, and the influence of dexamethasone, RU 486 and progesterone. *Eur J Endocrinol* 133, 527-33.

Brennand J.E, Calder A.A, Leitch C.R, Greer I.A, Chou M.M and MacKenzie I.Z. (1997) Recombinant human relaxin as a cervical ripening agent - The UK three-centre trial. *Br J Obstet Gynaecol* 104, 775-780.

Brennand J.E, Leask R, Kelly R.W, Greer I.A and Calder A.A. (1998) The influence of amniotic fluid on prostaglandin synthesis and metabolism in human fetal membranes. *Acta Obstet Gynecol Scand* 77, 142-150.

Brennand J.E, Leask R, Kelly R.W, Greer I.A and Calder A.A. (1998) Mechanisms involved in the stimulatory effect of amniotic fluid on prostaglandin production by human fetal membranes. *Prostaglandins Leukot Essent Fatty Acids* 58, 369-375.

Elliott C.L, Brennand J.E and Calder A.A. (1998) The effects of mifepristone on cervical ripening and labour induction in primigravidae. *Obstet Gynecol* 92, 804-9.

Chapter One

A Review of Parturition

1.1 Pregnancy and Parturition.

Parturition is a complex series of events which ideally should culminate in the delivery of a healthy baby to a healthy mother. In order for the process to be successful, physiological changes in the maternal genital tract must occur in synchrony, and at the appropriate gestation. Traditionally labour was thought to be entirely the result of contractility of the uterine corpus. However, recognition of the uterine cervix as a distinct entity, in which important physiological changes occur that are a prerequisite to normal labour, has altered this view. The function of the uterus and cervix changes as gestation advances. In early pregnancy the uterus is relatively quiescent while the cervix is a rigid structure, and it is the combination of these two features that prevents the untimely expulsion of the fetus. As pregnancy progresses, myometrial contractility increases and the cervix softens and becomes more compliant enabling it to dilate and allow the safe passage of the fetus. It can be seen, therefore, that the onset of labour is not a sudden event but rather the end point of a phase of increased myometrial responsiveness and cervical ripening, referred to as prelabour.

The mechanisms responsible for the control of parturition are understood only in part, and the exact stimulus for the onset of labour remains elusive. Any interruption to the fine tuning of events leading to the onset of labour can have important clinical implications, and it is for this reason that continuing research endeavour in to the control of parturition is essential on at least two counts. Preterm labour, which occurs in approximately 10% of pregnancies, is responsible for 85% of the deaths of normally formed babies. This remains one of the major clinical challenges in obstetric practise and our current management of this problem is based on our present knowledge about the process of parturition. Reducing the incidence of preterm labour is dependent on advancing our knowledge further. Apart from obvious concerns

about the timing of the onset of labour, it is widely accepted that the course of labour is least complicated when its onset is spontaneous. Clinical situations arise necessitating early termination of the pregnancy on account of maternal or fetal pathology, or intervention may be indicated owing to prolonged pregnancy. In these cases it is recognised that the success of labour induction is closely correlated with the cervical state (ie. ripeness), and that pharmacological cervical ripening / induction of labour should replicate physiological events as closely as possible.

The work presented in this thesis focuses on two areas. Firstly, the role of human fetal membranes in the production of potent myometrial contractile agents, namely prostaglandins, and factors which control their synthesis and metabolism. Secondly, the use of pharmacological agents for cervical ripening prior to induction of labour in women with an unfavourable cervix. In view of this, the physiology of parturition will be introduced.

1.2 Myometrial Contractility.

1.2.1 Physiology of Myometrial Contractility.

The uterus is never entirely quiescent, neither in the pregnant or the non-pregnant state. Caldeyro-Barcia and colleagues (1958) studied the pattern of myometrial contractility in pregnancy in detail, and demonstrated the presence of contractile activity throughout gestation, with an increase in both frequency and intensity of contractions in the 5-6 weeks prior to the onset of recognised "clinical labour".

The myometrium is composed of smooth muscle cells embedded in extracellular material consisting mainly of collagen fibres. It therefore differs in this respect from skeletal muscle which is a more homogeneous tissue. The smooth muscle cells are arranged in bundles and exist in two distinct layers: outer longitudinal and inner circular according to the configuration of the bundles. Communication between the cells is facilitated by the collagenous ground substance and by gap junctions.

Gap Junctions.

Gap junctions facilitate intercellular communication which has been shown to be important in a number of physiological processes including the coordination of smooth muscle contraction (Garfield et al, 1977). They consist of two symmetrical portions of plasma membrane from apposing cells. Intramembranous proteins (connexins) from these apposing cells align themselves, creating pores from the cytoplasm of one cell to the other. These pores provide sites for low-resistance electrical or ionic coupling between cells and are thought to synchronise myometrial contractility by conduction of electrophysiological stimuli during labour.

There are at least 16 types of connexin gap junction proteins, all of which share the same general structure (Orsino et al, 1996). However, the pattern of distribution of connexins is complex: some are broadly expressed in many tissues (eg. connexin 43 [Cx-43]), and others show a restricted pattern of distribution (eg. Cx-33). Cx-43 is the major myometrial gap junction protein (Lye et al, 1993). It has been demonstrated that the expression of myometrial Cx-43 messenger RNA is elevated near term and is maximal during delivery in the rat (Lye et al, 1993), sheep (McNutt et al, 1994) and human (Chow and Lye, 1994).

Gap junction regulation.

The fact that expression of myometrial Cx-43 mRNA is elevated at term and in association with labour suggests that regulation of gap junction proteins may depend, at least in part, on hormonal steroid concentrations. In the rat and sheep Cx-43 expression is associated with an increase in the plasma oestrogen: progesterone ratio (Lye et al, 1993; McNutt et al, 1994). Investigation into the regulation of transcription of the Cx-43 gene in myometrium in animal models has demonstrated that this gene is oestrogen responsive, and that oestrogen increases mRNA encoding the nuclear transcription factors Fos and Jun. Therefore, oestrogen could stimulate transcription of the Cx-43 gene directly via putative oestrogen responsive elements or indirectly via increased expression of c-fos or c-jun (Lefebvre et al, 1995). The finding that incubation of bovine myometrial cells with the antioestrogen EM 139 inhibits Cx-43 mRNA expression by up to 40% (Doualla-Bell et al, 1995) lends further support to the theory that steroids are important regulators of gap junctional proteins and hence myometrial contractility.

An increase in the oestrogen: progesterone ratio will therefore favour induction of the Cx-43 gene and hence positively influence myometrial contractility. Progesterone can block oestrogen-induced expression of Cx-43 mRNA (Petrocelli and Lye, 1993) raising the possibility that there may be progesterone response elements in the Cx-43 gene. However, whether progesterone withdrawal itself has a positive effect on gap junction formation, or whether this is simply secondary to the relative increase in oestrogen concentrations that occurs as a result has not been established.

The mechanisms that regulate gap junction synthesis and function have not been identified in humans. There is no change in peripheral progesterone concentrations in humans prior to the onset of labour suggesting that any change in

oestrogen/progesterone ratio that might regulate gap junction formation would have to occur at a local level. It has been suggested that it is not the level of steroid hormones that is important in regulating Cx-43 expression, but rather the concentration of myometrial steroid receptors (Chow and Lye, 1994). It has been demonstrated that an increase in mRNA encoding myometrial oestrogen receptors may occur with labour onset, enabling increased oestrogen activity (with a change in the oestrogen/progesterone ratio) which would not be reflected in the peripheral circulation (Mitchell et al, 1982).

Myosin and Actin.

Myosin and actin filaments within the smooth muscle cell are central to the process of muscle contraction. Myosin differs from actin in that in addition to being a structural protein, which is organised in thick filaments, it is also an enzyme responsible for hydrolysing ATP during contraction and relaxation. Myosin is composed of one pair of heavy chains of about 200 kDa and two pairs of light chains of 15 and 20 kDa. The head of the myosin molecule has three important sites: the actin combining site; the ATPase site and the 20 kDa light chains. The actin-combining site is where myosin interacts with actin, the other major muscle protein. It is a globular protein of 45 kDa, organised into thin filaments. Smooth muscle contraction occurs when covalent cross-linking bonds form between the actin and myosin filaments as they slide past each other. Whereas in skeletal muscle the direction of contraction is always aligned with the axis of the muscle fibres, owing to the organisation of the filaments in smooth muscle it is able to exert pulling forces in any direction.

1.2.2 Regulation of Myometrial Contractility.

Myosin light chain kinase (MLCK).

MLCK is the enzyme responsible for phosphorylation of the 20 kDa myosin light chains. This reaction results in the release of energy required to form the cross-linking bonds between the actin and myosin filaments. MLCK therefore has an integral regulatory role in smooth muscle contractility. The enzyme itself is controlled by a number of factors. Activation of MLCK is effected by phosphorylation of the enzyme, brought about by calmodulin. Calmodulin is a calcium-binding protein, which may in fact be part of the MLCK molecule itself, which on binding to calcium alters its steric properties enabling it to phosphorylate MLCK. Clearly, calcium is also a pivotal regulator of MLCK activity and will be discussed in more detail below.

Second messengers.

Another mechanism by which MLCK activity is modulated is via adenosine 3,5-monophosphate, commonly known as cyclic AMP (cAMP). This nucleotide acts as a second messenger in the action of many hormones. It is formed from ATP by the action of adenylate cyclase, a membrane-bound enzyme. Adenylate cyclase is activated when a hormone binds to its specific receptor on the cell membrane, and the result is increased concentrations of cAMP within the cell. The cAMP then acts within the cell to alter the rate of one or more processes.

In the case of smooth muscle contractility, cAMP can stimulate the calcium pump of the sarcoplasmic reticulum which leads to a reduction in cytoplasmic concentrations of free calcium, resulting in muscle relaxation. In addition, cAMP can activate cAMP-dependent-protein kinase A, by binding to its regulatory subunit. Activated protein

kinase A is then able to phosphorylate MLCK, this time reducing its ability to bind calmodulin and thereby inhibiting uterine contractility. Alternatively, activated protein kinase A can phosphorylate a membrane binding site for calcium resulting in decreased intracellular calcium concentrations.

It must be remembered that MLCK has two sites available for phosphorylation: one leading to activation of the enzyme, and the other resulting in its inhibition. There is, therefore, a complex chain of events surrounding the regulation of myometrial contractility, and potential for its modulation by various hormones and pharmacological agents. Stimulation of adenylate cyclase will result in myometrial relaxation through increased cAMP concentrations, as will inhibition of the enzyme responsible for cAMP degradation, phosphodiesterase.

Calcium.

In human myometrium the extent of myosin light chain phosphorylation correlates strongly with the intracellular free calcium concentration (MacKenzie et al, 1990). The major intracellular store of calcium is the sarcoplasmic reticulum (Somlyo et al, 1985), the membranes of which contain an intracellular ATP-dependent calcium transport pump which can facilitate large variations in intracellular free calcium concentrations. Release of calcium from the sarcoplasmic reticulum can be promoted by inositol 1,4,5-triphosphate (IP_3), another second messenger, which is produced as a result of the action of phospholipase C on the membrane glycerophospholipid phosphatidylinositol. In addition to the intracellular sources of calcium, extracellular calcium can enter the cytoplasm, following the appropriate stimulation, to increase free calcium concentrations. There are two main routes for calcium entry: voltage and receptor-operated channels (Hurwitz, 1986). The voltage-operated channels depend on membrane depolarisation to a threshold level at which the channels become activated

and allow influx of calcium to the cell. Receptor-operated channels, on the other hand, become activated and open in response to ligands such as hormones and neurotransmitters.

1.2.3 Hormonal regulation of myometrial contractility.

Progesterone.

Progesterone is traditionally regarded as the hormone responsible for the maintenance of uterine quiescence throughout gestation (Csapo, 1975). Potential mechanisms for this include uncoupling of the excitation-contraction process and inhibition of prostaglandin or gap junction formation. Uncoupling of the excitation-contraction process can be effected by altering membrane permeability or influencing postreceptor events. Progesterone promotes membrane-binding of calcium, and sequestration of calcium within the sarcoplasmic reticular pools, thus reducing intracellular free calcium concentrations (Currie and Jeremy, 1979). This is in part due to an action on the myometrial cell membrane phospholipids, inhibiting release of phosphatidylinositol and the second messenger IP_3. A post-receptor effect has been demonstrated by work on the rabbit showing an increased response to β-adrenergic receptor stimulation in the presence of progesterone, and that the rate of cAMP synthesis was much greater under the influence of progesterone compared with oestrogen (Riemer et al, 1986).

Progesterone is capable of inhibiting both calcium ionophore- and arachidonic acid-stimulated production of prostacyclin (PGI_2) and thromboxane (TXB_2) in nonpregnant rat myometrium (Jeremy and Dandona, 1986). Since both of these prostaglandins may be involved in uterine contractility in the rat (Williams et al, 1979) it has been

proposed that progesterone exerts some of its quiescent effect by inhibiting endogenous prostaglandin synthesis.

Oestrogens.

During late pregnancy oestrogens promote uterine contractility in most species. They are essential for the induction of protein synthesis in the uterus, particularly the contractile proteins (Csapo, 1969) and are capable of increasing myometrial sensitivity to a variety of oxytocic agents, for example oxytocin and $PGF_{2\alpha}$. This is likely to be the result of an increase in receptor synthesis, and there is evidence from several studies to suggest that prostaglandin and oxytocin receptor concentration is under the control of ovarian hormones (Soloff et al, 1977). Administration of oestrogen will increase the number of uterine receptors sensitive to oxytocin and α-adrenergic agonists (Nissenson et al, 1978; Roberts et al, 1977) and this increase can be blocked by the simultaneous administration of progesterone.

In addition to increasing receptor synthesis, oestrogen has been shown to increase prostaglandin synthesis and release from human uterine tissue (Thorburn and Challis, 1979). These events in part explain the stimulatory effect of oestrogen on uterine contractility. Finally, oestrogen can also influence intracellular calcium concentrations, calmodulin and MLCK activity (Matsui et al, 1983).

Oxytocin.

Oxytocin is a posterior pituitary hormone which when bound to its receptor inhibits the myometrial cell membrane calcium ATPase, responsible for pumping calcium to the extracellular environment. In addition, calcium influx from both the sarcoplasmic reticulum and the extracellular area is promoted. These actions result in an increase in

intracellular free calcium concentrations which as previously discussed favours the contractile process. High affinity oxytocin receptors are present in human myometrium and increase in concentration towards term (Fuchs et al, 1984). Oxytocin receptors appear at the same time as gap junctions and it has been proposed that they may be important in regulating the function of these gap junctions (Garfield and Beier, 1989). As well as being present in myometrial cells, oxytocin receptors have been demonstrated in the decidua parietalis at term (Fuchs et al, 1984). In addition oxytocin mRNA has been demonstrated in amnion, chorion and decidua, with levels being highest in decidua (Chibbar et al, 1995). Oxytocin gene expression was 3-4 fold higher in choriodecidual tissue collected following spontaneous labour. The production of decidual oxytocin has importance with regard to stimulating contractility in the adjacent myometrium. Also, since the decidua is an important site for prostaglandin synthesis, production and binding of oxytocin to its receptor may influence prostaglandin production. Finally, oxytocin stimulates the phosphatidylinositol pathway (Molnar and Hertelendy, 1990) leading to the release of the second messenger IP_3 which in turn increases intracellular calcium concentrations.

Adrenergic drugs.

Human myometrium contains α_1, α_2 and β_2 adrenoreceptors, and these effect either smooth muscle contraction or relaxation depending on their interaction with the regulatory mechanisms discussed above. β_2 receptors stimulate cAMP (Litime et al, 1989) and hence muscle relaxation, providing the rationale for the use of β agonists in the prevention of preterm labour. The stimulatory effect of β agonists on cAMP formation appears to be maximal prior to 35 weeks gestation, and is subsequently lost as pregnancy advances to term (Litime et al, 1989). This is thought to represent uncoupling of the β_2 receptor to adenylate cyclase rather than a decrease in receptor number.

β_2 receptors may promote smooth muscle relaxation by a mechanism independent from cAMP. In guinea-pig myometrium β-adrenergic receptor activation is linked to inhibition of voltage-operated calcium channels (Khac et al, 1992), and inhibition of these channels prevents phospholipase C activation and thus inhibits IP_3 release. α_1 receptors are linked to phospholipase C (Breuiller-Fouche et al, 1991) and hence stimulate uterine contractility via IP_3. α_2 receptors have dual function: they can stimulate uterine contractions by inhibiting adenylate cyclase, resulting in decreased cAMP concentrations (Breuiller et al, 1990), or they can inhibit calcium channels leading to uterine relaxation.

Prostaglandins.

Prostaglandins (section 1.3) are capable of stimulating myometrial contractility throughout pregnancy. The pregnant uterus is more sensitive than the non-pregnant, but it is not clear whether there is increasing sensitivity with advancing gestation. Prostaglandins are not universally uterotonic, and it is the balance between stimulation and inhibition that is likely to be important in regulating uterine activity throughout pregnancy and labour. They exert their effects by binding to specific receptors located in the plasma membranes of myometrial cells. There appears to be no change in receptor number or affinity in association with labour, but rather, the increased myometrial contractility is a direct result of increased prostaglandin concentrations (Huszar and Naftolin, 1984).

There are three different types of PGE receptor in the human uterus (Coleman, 1991): EP_1 and EP_3 receptors are stimulatory, while EP_2 receptors are inhibitory. This helps to explain the *in vitro* effects of PGE_2 which at low doses is stimulatory, but at high doses has a biphasic action of rapid contraction followed by prolonged relaxation. EP_1 and EP_3 receptors both activate phosholipase C, and EP_3 receptors also inhibit

cAMP formation. EP_2 receptors exert their effect by generating cAMP. Prostaglandin $F_{2\alpha}$ is purely stimulatory and binding to its specific receptor results in activation of phospholipases C and A_2 (Molnar and Hertelendy, 1990).

The stimulatory receptor interactions result in a final common event which is an increase in intracellular free calcium. This occurs either as a result of increased transport from the extracellular space, or release from intracellular stores. Calcium is not only essential for smooth muscle contractility, but is also required for prostaglandin synthesis. Incubation of cell cultures with calcium channel blockers will inhibit prostaglandin synthesis, whereas calcium ionophores lead to increased production (Olson et al, 1983a). There are therefore two mechanisms which make calcium channel blockers potentially useful in the arrest of preterm labour. Other prostaglandins (and their receptors) that influence myometrial contractility include PGI_2 (prostacyclin), which is inhibitory as a result of increased adenylate cyclase activity (Tanfin and Harbon, 1987), and thromboxane which is stimulatory via phospholipase C (Hirata et al, 1991). In addition to their post receptor effects prostaglandins can modulate gap junction formation *in vitro* (Garfield et al, 1980). Prostaglandins E_2 and $F_{2\alpha}$ induce gap junction formation, while others such as PGI_2 are inhibitory. There is therefore a balance between stimulatory and inhibitory prostaglandins and their receptors within the myometrium which is likely to be important in the regulation of uterine activity. In addition, the location of these receptors in different regions of the uterus may be central to the facilitation and coordination of uterine contractility.

1.3 Prostaglandin Biochemistry.

Prostaglandins are thought by many to be central to the process of parturition (section 1.4) and therefore basic principles of prostaglandin biochemistry will be discussed in the following section.

1.3.1 Nomenclature and Structure.

The chemical structure of the naturally occurring prostaglandins was elucidated by Nugteren et al, 1966. They are oxygenated, unsaturated hydroxy fatty acids containing 20 carbon atoms. The underlying structure of all prostaglandins is the prostanoic acid molecule which consists of a cyclopentane ring with two aliphatic side chains (Figure 1.1). Traditionally, the carbon skeleton is numbered consecutively from the terminal carboxyl function. The individual prostaglandins are characterised by the number of substituents on different carbon atoms, and the structure of the cyclopentane ring. Prostaglandins E (ether soluble) and F (fosphate soluble [Swedish spelling]) were the first to be named, followed by prostaglandins A, B, C, D, G, H and I. Prostaglandins contain one or more double bonds, the number of which is indicated by a numerical subscript (1-3) which follows the corresponding letter referring to the parent structure. All prostaglandins contain a 13,14-trans double bond, while the presence of a second double bond at the 5,6 position, and a third one at the 17,18 position characterises prostaglandins of the 2 and 3 series respectively. Prostaglandins of the F series have an additional subscript (α or β) which denotes the orientation of the hydroxyl group at C-9. The cyclic endoperoxides prostaglandins G_2 and H_2 are short-lived intermediates in the biosynthetic pathway leading to the

formation of the classical prostaglandins, and prostaglandin I_2 (prostacyclin) and thromboxane (TXA_2) have ring configurations which differ from the basic structures of the other prostaglandins. The structures of the cyclic endoperoxides and the common prostaglandins are illustrated in Figure 1.2.

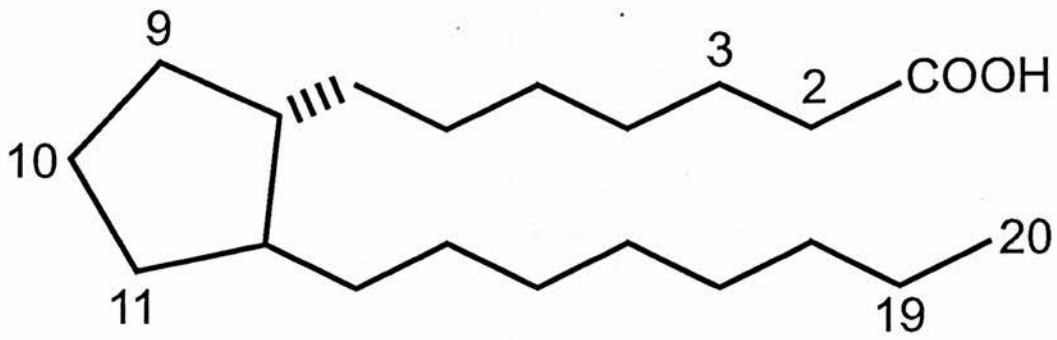


Figure 1.1
Hypothetical "Prostanoic Acid" skeleton

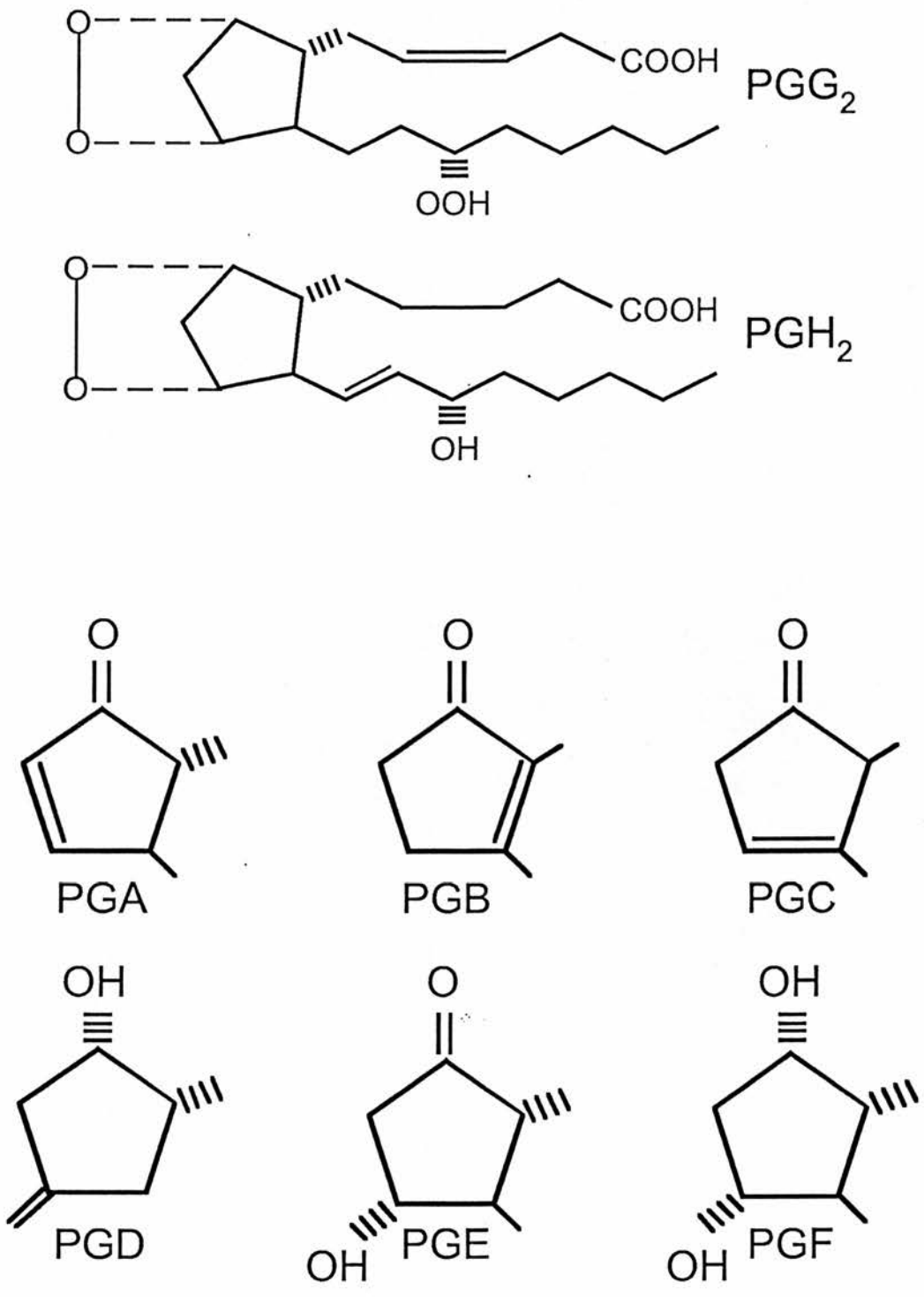


Figure 1.2
Structures of the cyclic endoperoxides (PGG_2, PGH_2), and the cyclopentane rings of common prostaglandins.

1.3.2 Prostaglandin Biosynthesis.

All naturally occurring prostaglandins are synthesised from one of three essential 20 carbon unsaturated fatty acids. Arachidonic acid (5, 8, 11, 14-all-cis-eicosatetranoic acid) is the most important and leads to the synthesis of prostaglandins of the 2 series. The other two are 8, 11, 14-all-cis-eicosatrienoic acid and 5, 8, 11, 14, 17-all-cis-eicosapentanoic acid which give rise to prostaglandins of the one and three series respectively. Arachidonic acid is present in cells in the esterified form, usually in the sn2 position of the glycerophospholipid. It is the liberation of arachidonic acid that is thought to be the rate-limiting step in eicosanoid biosynthesis (Kunze and Vogt, 1971).

The release of arcahidonic acid from glycerophospholipids is accomplished either by the direct action of the enzyme phospholipase A_2, or by the sequential actions of phospholipase C, monoacylglycerol and diacylglycerol lipases. The liberated arachidonic acid can then be metabolised by at least three major pathways (Figure 1.3) resulting in formation of prostaglandins, hydroxyeicosatetraenoicacids or leukotrienes. The pathway leading to prostaglandin synthesis involves the conversion of arachidonic acid to the key intermediates prostaglandin G_2 and then prostaglandin H_2 by the action of the enzyme fatty acid cyclooxygenase (COX, also known as prostaglandin endoperoxide synthase [PGHS]) which contains an inherent peroxidase activity. Two isoenzymes of COX are known to exist and are discussed in section 1.5.2. Prostaglandins G_2 and H_2 thus formed can be converted either non-enzymatically to PGE_2, or enzymatically to PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 depending on the cell type in which biosynthesis is occurring (Samuelsson et al, 1975).

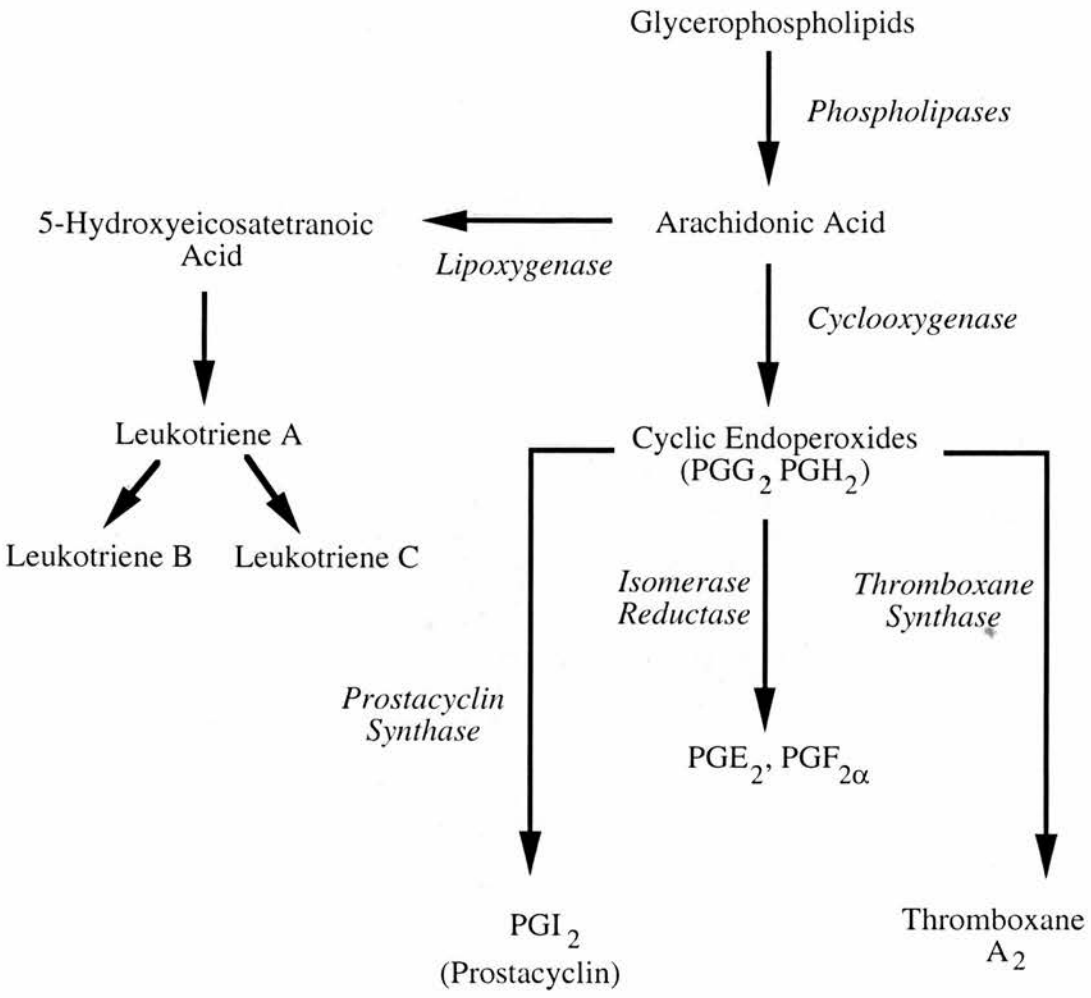


Figure 1.3 The Arachidonic Acid Cascade

Prostaglandin biosynthesis can be inhibited by a number of different agents. The most important group is the non steroidal anti-inflammatory drugs (NSAIDs) which inhibit the action of COX. It has been demonstrated that the mode of action of NSAIDs differs according to the preparation. Aspirin causes irreversible inhibition of COX, where recovery of the enzyme is dependent on new protein synthesis, whereas other drugs such as diflunesal act as reversible inhibitors (Lands and Hanel, 1983). Structural analogues of arachidonic acid, such as arachidynoic acid, will act as competitive inhibitors of prostaglandin synthesis (Ahern and Downing, 1970), and anti-inflammatory steroids will inhibit the release of precursor acids by inhibiting phospholipase A_2.

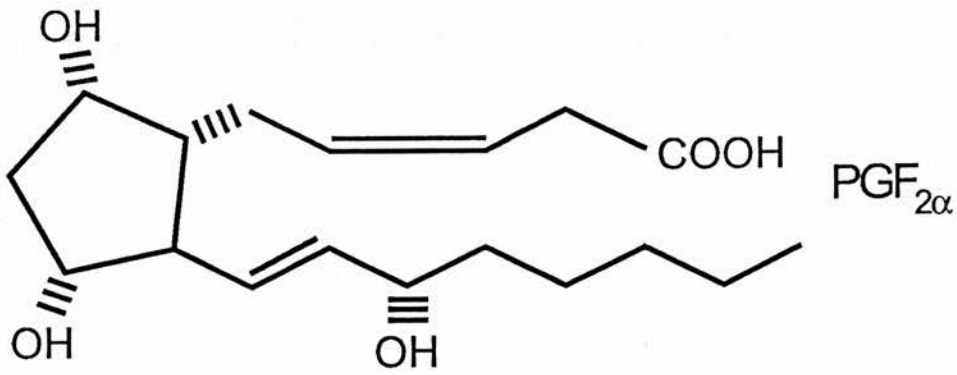
1.3.3 Prostaglandin Metabolism.

Prostaglandin metabolism takes place mainly in the lung, but the enzymes involved in this process are also found in the kidney and liver (Anggard et al, 1971). In pregnancy chorion is the major site of intrauterine prostaglandin metabolism and since this thesis concentrates on the metabolism of prostaglandins E and F in relation to parturition, they will be discussed more specifically here. However, the following description does apply, in general, to all prostaglandins.

The first step in prostaglandin metabolism is the oxidation of the allylic alcohol group at the site of carbon-15 by the enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH). This reaction yields 15-keto-prostaglandins which are biologically inactive compounds. Following this, reduction of the Δ-13-double bond leads to formation of 15-keto-13,14-dihydro-prostaglandins, the metabolites which are found in the circulation. These metabolites have a circulating half-life of approximately 8 minutes,

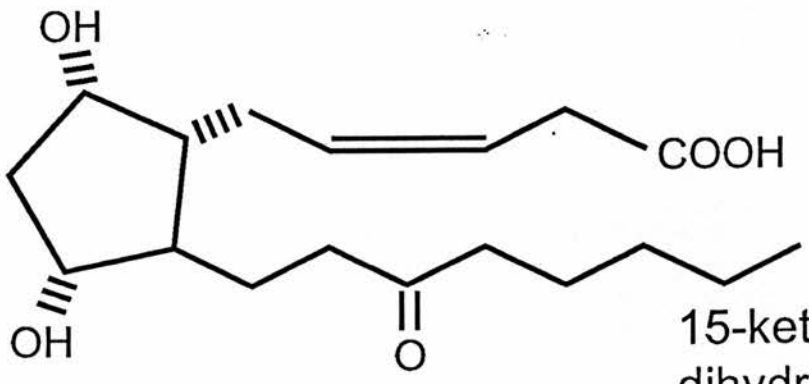
unlike their parent prostaglandins which have an estimated half-life of less than 15 seconds in the circulation. Because of their short half-life, and the fact that platelets, which are invariably activated on plasma collection, synthesise PGE_2 and $PGF_{2\alpha}$ (Samuelsson et al, 1975), estimation of primary prostaglandins in circulation is of no value. In contrast, estimation of stable prostaglandin metabolite concentrations can be used to monitor changes in prostaglandin biosynthesis.

The 15-keto-13,14-dihydro-prostaglandins undergo further metabolic degradation to yield metabolites with 18, 16 and 14 carbon atoms which are the major urinary metabolites (Samuelsson et al, 1975). This involves a series of β and ω oxidation reactions. β oxidation removes 2 carbon atoms from the carboxyl side-chain and one or two steps of β oxidation will result in di-nor or tetra-nor compounds respectively. ω oxidation at the ω end of the molecule gives rise to a hydroxyl or carboxyl group at this site, which also undergoes β oxidation yielding ω dinor and ω tetranor dioic acid compounds. As a result of these reactions the major urinary metabolites of PGE_2 and $PGF_{2\alpha}$ are 7α-hydroxy-5,11-diketotetranorprostane-1,16-dioic acid and 5α,7α-dihydroxy-11-ketotetranorprostane-1,16-dioic acid respectively. A simplified metabolic pathway for $PGF_{2\alpha}$ is illustrated in Figure 1.4.



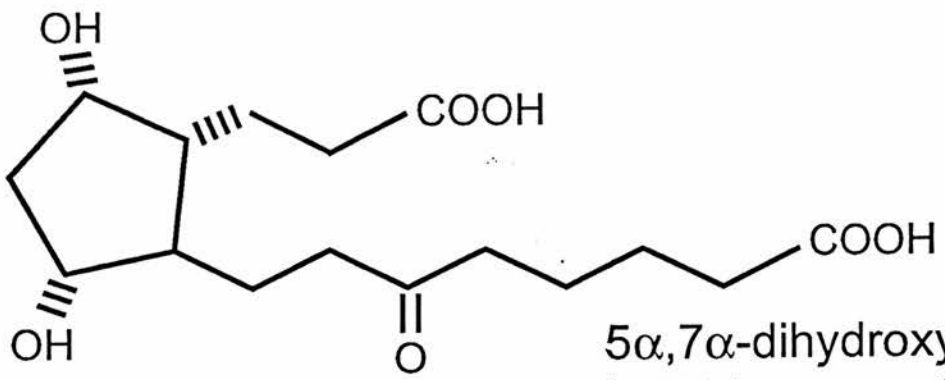
$PGF_{2\alpha}$

PLASMA METABOLITE



15-keto-13,14-dihydro-$PGF_{2\alpha}$

URINE METABOLITE



5α,7α-dihydroxy-11-ketotetranorprostane-1,16-dioic acid

Figure 1.4
Simplified metabolic pathway for $PGF_{2\alpha}$.

1.4 Prostaglandins and Parturition.

There is a large body of evidence to support a role for prostaglandins in human parturition. A number of prostaglandins were identified in amniotic fluid by Karim (1966), who also demonstrated that prostaglandin-rich extracts of amniotic fluid were capable of increasing the intensity of spontaneous myometrial contractions (Karim and Devlin, 1967). In addition, concentrations of PGE and PGF were significantly greater in amniotic fluid obtained following spontaneous labour compared with those found in amniotic fluid collected prior to labour onset (Karim and Devlin, 1967). These findings have been confirmed by other workers in this field (Keirse et al, 1977a; Mitchell et al, 1979). Prostaglandin metabolite concentrations are also increased during labour, and the approximate increase in the prostaglandins and their metabolites are as follows: PGE_2 10-fold, $PGF_{2\alpha}$ 12-fold (Dray and Friedman, 1976), 13,14-dihydro-15-keto-$PGF2_{\alpha}$ 6-fold (Keirse et al, 1977a), 6-keto-$PGF1_{\alpha}$ 2-fold (Mitchell et al, 1979) and thromboxane B_2 4-fold (Mitchell et al, 1978a). Similarly, prostaglandin metabolite concentrations in maternal plasma are increased in association with labour (Mitchell et al, 1978b; Johnston et al, 1993). Urinary metabolite concentrations have also been studied during pregnancy and the daily excretion of PGFM increases with advancing gestation, and doubles just prior to labour onset (Hamberg, 1974).

Prostaglandins are known to be successful in inducing labour at all stages of pregnancy (Novy and Liggins, 1980), and chronic administration of the NSAID aspirin, which inhibits COX and hence the conversion of arachidonic acid to the cyclic endoperoxides, results in prolongation of gestation (Collins and Turner, 1975). Likewise, administration of indomethacin following induction of midtrimester abortion will prolong the induction-to-abortion interval (Waltman et al, 1972).

1.5 Fetal Membranes and Prostaglandin Biosynthesis.

The fetal membranes and decidua vera are an important source of the prostaglandins involved in parturition. All three tissues, amnion, chorion and decidua, contain PGHS activity and while PGE_2 is the major product of the amnion and chorion, decidua produces PGE_2 and $PGF_{2\alpha}$ in equal amounts (Mitchell et al, 1978c; Okazaki et al, 1981a). PGHS activity is greater in amnion compared with chorion and decidua, and it is significantly greater in amnion collected following spontaneous labour compared with pre-labour (Okazaki et al, 1981a). Although the precise source of the prostaglandins integral to the initiation of labour has not been clarified, the increased production of PGE_2 by amnion is thought to be important in a complex cascade of events.

Since amnion is avascular, substances that influence prostaglandin production by this tissue are likely to be present locally, either in the amniotic fluid, chorion laeve or decidua. The theory that the fetus may play a role in controlling the timing of parturition is an attractive one. As will be discussed later there is some evidence to support interaction between the placental unit and the hypothalamo-pituitary-adrenal (HPA) axis of the fetus. Maturation of the fetal HPA axis may influence the content of the amniotic fluid, which in turn could regulate prostaglandin production by the fetal membrane and thus effect myometrial contractility (MacDonald et al, 1974). Because of the crucial role of the fetal membranes and decidua in this cascade the previous account of prostaglandin biosynthesis and metabolism, and the regulation therof, will now be related to these tissues.

1.5.1 Arachidonic Acid.

As discussed earlier unesterified arachidonic acid is the obligate precursor of prostaglandins of the 2-series (Lands and Samuelsson, 1968) and release of arachidonic acid from esterified forms may be a rate limiting step in prostaglandin synthesis. The concentration of arachidonic acid in amniotic fluid increases 6-10 fold during labour (MacDonald et al, 1974) and this increase is 4 times greater than that of other unesterified fatty acids. In addition, the concentration of arachidonic acid in amniotic fluid has been shown to be related to cervical dilatation (Keirse et al, 1977b).

Arachidonic acid accounts for approximately 20% of total fatty acids in amniochorion (Schwarz et al, 1975), and the arachidonic acid content of fetal membranes is significantly lower in tissue obtained after labour compared with that collected pre-labour. Over 45% of the arachidonic acid in glycerophospholipids is present in phosphatidylethanolamine (PE) (Okita et al, 1982a). The arachidonic acid content of PE, and another glycerophospholipid phosphatidylinositol (PI), is reduced by about 42% and 35% respectively in amnion during early labour (Okita et al, 1982a). Smaller but significant reductions in the arachidonic acid content of these glycerophospholipids were also seen in chorion.

Enzymatic Release of Arachidonic Acid.

Phospholipase A_2

Phospholipase A_2 (PLA_2) is capable of releasing arachidonic acid esterified at the sn-2 position of glycerophospholipids. PLA_2 activity has been demonstrated in a number of intrauterine tissues (Schultz et al, 1975) and in amniochorion it is largely distributed

in microsomal and cytosolic fractions (Okazaki et al, 1978). PLA_2 has been shown to be substrate specific in amniochorion, promoting the release of arachidonic acid from PE, but not from PI, and in general its activity is calcium dependent (Okazaki et al, 1978).

Multiple forms of PLA_2 have been identified and characterised into two functionally distinct groups: secretory PLA_2s and cytosolic PLA_2. Messenger RNAs encoding two secretory PLA_2 isoenzymes (Types II and IV) have been identified and expression of these mRNA transcripts and that of cytosolic PLA_2 has been investigated in the intrauterine tissues at term. Cytosolic PLA_2 mRNA is most abundant in amnion, compared with choriodecidua or placenta, whereas secretory mRNA transcripts are most abundantly expressed in placenta (Freed et al, 1997). These findings suggest that cytosolic PLA_2 is the principal isoenzyme mediating arachidonic acid release in term amnion, whereas secretory PLA_2 isoenzymes are integral to placental phospholipid metabolism.

Phospholipase C

Phospholipase C (PLC) is also a calcium-dependent enzyme, and a PI-specific phospholipase C has been isolated in amnion, chorion and decidua (Di Renzo et al, 1981). PLC will not itself release unesterified arachidonic acid from PI, but leads to the production of diacylglycerol (DAG) rich in arachidonic acid. The diacylglycerol content of human amnion is greater in tissue obtained during early labour compared with that obtained prelabour, supporting the theory that arachidonic acid is released from PI in amnion during parturition (Okita et al, 1982b). DAG is then hydrolysed at the sn-1 position by the action of DAG lipase which is present in fetal membranes and decidua (Okita et al, 1982b). This reaction results in monoacylglycerol (MAG) which is subsequently hydrolysed by monoacylglycerol lipase to release the fatty acid in the sn-2 position which is rich in arachidonic acid (Okazaki et al, 1981b).

Regulation of Arachidonic Acid Mobilisation.

It has been demonstrated *in vitro* that the activities of PLA_2 and PLC increase during gestation, and that this increase is confined to amnion (Okazaki et al, 1981c). This is unlike the activities of DAG lipase and MAG lipase which remain constant throughout gestation. In spite of the gestational changes in PLA_2 and PLC activity, there is no further change in the activity of these enzymes in fetal membranes or decidua vera in association with labour. Both PLA_2 and PLC are calcium dependent, and the calcium concentration necessary to support maximal activity of these enzymes *in vitro* is greater than that found in amnion cells under resting conditions. Factors present in amniotic fluid may have the potential to increase calcium concentrations in amnion cells. Platelet activating factor (PAF), which has been identified in amniotic fluid (Billah and Johnston, 1983), is able to induce rapid increases in intracellular calcium concentrations leading to activation of phospholipases, arachidonic acid release and prostaglandin production (Roukin et al, 1983). PAF will also stimulate PGE_2 production from amnion tissue culture (Di Renzo et al, 1984).

1.5.2 Cyclooxygenase.

Cyclooxygenase (COX), otherwise known as prostaglandin endoperoxide H synthase (PGHS), protein has been identified in human fetal membranes and decidua from early gestation to term by immunocytochemistry (Bryant-Greenwood et al, 1987). COX catalyses the conversion of free arachidonic acid to the unstable cyclic endoperoxides, which are in turn converted to prostaglandins, prostacyclin or thromboxane depending on the cell type. The activity of this enzyme is therefore an important rate-limiting step in prostaglandin biosynthesis. Two isoenzymes of COX have been identified, and each one is the product of a different gene (Xie et al, 1993; Wen et al, 1993). COX-1

is a 68.5 kDa protein which is constitutively expressed in fetal membranes, and *in situ* hybridisation has localised the expression of this isoenzyme to the amniotic epithelium, amniotic mesoderm and chorionic layers (Slater et al, 1995). COX-2 is the inducible form of the enzyme, weighing 69 kDa, and its expression can be stimulated by various agents including cytokines (Mitchell et al, 1993a). It has been localised specifically to the epithelial and mesodermal layers of the amnion with very little expression in chorion (Slater et al, 1995).

COX activity in amnion is known to increase with advancing gestation, with a further increase in association with the onset of labour (Teixeira et al, 1993). In addition, a 4 fold increase in COX activity in amnion collected from idiopathic preterm labour has been demonstrated, suggesting that increased COX activity is a common factor to both preterm and term labour (Teixeira et al 1994). Investigation of mRNA levels for COX-1 and 2 in human amnion has revealed that COX-2 mRNA, but not COX-1 mRNA, is increased in association with labour onset, suggesting that increased COX-2 expression is responsible for the increased COX activity seen at this time (Hirst et al, 1995). COX-1 has a short half life of approximately 4 minutes, and COX-2 appears to have similar kinetics (Xie et al, 1992). Therefore, if COX activity is to be maintained there must by continuous enzyme synthesis, and this rate of synthesis will have to increase if enzyme activity is to be enhanced. Based on these findings COX-2 activity is thought to be of principal importance in producing the increase in prostaglandin concentrations seen with the onset of labour.

Regulation of COX.

A number of agents have been shown to stimulate *de novo* synthesis of COX, including epidermal growth factor (EGF), interleukin-1 (IL-1), dexamethasone and TPA (Olson et al, 1990). The effect of corticosteroids on prostaglandin production

does vary, however, with the type of culture employed. For example, while glucocorticoids stimulate PGE_2 synthesis in confluent cell cultures, they inhibit prostaglandin synthesis in dispersed cell or explant cultures (Gibb and Lavoie, 1990). Cortisol has been shown to reduce the amount of immunoreactive PGHS in amnion tissue (Zakar et al, 1994), and in the same experiments TPA, an activator of protein kinase C, stimulated COX activity. Contrary to these findings, glucocorticoids can increase the level of COX-2 mRNA in confluent primary amnion cell cultures, resulting in increased conversion of arachidonic acid to PGE_2 in these cells (Zakar et al, 1995).

1.5.3 Protein Kinase C.

Protein kinase C (PKC) is a calcium and lipid-dependent protein kinase which regulates cellular function. Activation of PKC occurs as a result of increased intracellular diacylglycerol (DAG) concentrations. DAG binds to PKC and in so doing increases the enzymes affinity for calcium (Nishizuka, 1988). PKC can therefore become fully activated without a detectable change in intracellular calcium concentration (Nishizuka, 1988). PKC activity has been demonstrated in amnion (Okazaki et al, 1984) and activation of this enzyme is associated with the increased prostaglandin synthesis seen with the onset and maintenance of labour (Olson et al, 1990). This process of activation, which involves phosphorylation, appears to require DAG, phosphatidylserine and calcium (Okazaki et al, 1984).

It has been proposed that PKC is involved in arachidonic acid mobilisation, and the activation of COX via RNA and protein synthesis-dependent mechanisms (Zakar and Olson, 1988). PKC exists in multiple isoforms (Nishizuka, 1988), and a number of

stimulants and inhibitors of the enzyme have been identified. Phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) and 12-O-teradecanoylphorbol-13-acetate (TPA), are tumor-promoting agents which are structurally similar to DAG and are capable of activating PKC. They have been shown to stimulate prostaglandin production from amnion, chorion and decidua (Sander and Myatt, 1990; Lundin-Schiller and Mitchell, 1991a; Schrey et al, 1987). Phorbol ester stimulation of amnion PGE_2 production is dependent on mRNA and new protein synthesis. Cycloheximide, an inhibitor of translation, will inhibit TPA-stimulated PGE_2 production in a dose-dependent manner. Actinomycin D, an inhibitor of transcription, has a similar effect (Zakar and Olson, 1988). These findings suggest that phorbol esters may induce *de novo* synthesis of enzymes involved in prostaglandin synthesis. This is further supported by the finding that pretreatment of amnion cells with acetylsalicylic acid does not affect subsequent TPA stimulation of PGE_2 production (Zakar and Olson, 1988), implying that the protein kinase C-mediated effect of the phorbol ester results in *de novo* synthesis of COX.

Prolonged exposure to phorbol esters will, however, result in down regulation of PKC and abolish or reduce PGE_2 production by amnion exposed to stimulatory agents such as oxytocin (Moore and Moore, 1990). Staurosporine is a PKC inhibitor which will completely inhibit the stimulation of PGE_2 production induced by agents such as EGF, dexamethasone, oxytocin and phorbol esters (Moore and Moore, 1990). The effect of interleukin-1β (IL-1β), another stimulant of amnion PGE_2 production, is also attenuated by PKC down regulation (Mitchell et al, 1993b). However, it has also been shown that staurosporine treatment can enhance PGE_2 stimulation by IL-1β (Mitchell et al, 1994). The regulation of PKC is clearly complex, and the diverse actions of this enzyme may reflect the differential responsiveness of its various isoforms.

As well as having a regulatory role in COX activity, PKC may also be involved in control of arachidonic acid release. As previously stated, activation of PKC leads to phosphorylation of a number of proteins, which results either in their activation or inhibition. Lipocortin is an antiphospholipase A_2 protein which is present in amnion (Mitchell et al, 1988a). Phosphorylation of lipomodulin, a lipocortin-like protein, reduces its ability to inhibit PLA_2 (Hirata, 1981). Activation of PKC may therefore, by phosphorylating endogenous lipocortin and thus reducing PLA_2 inhibition, increase arachidonic acid release.

1.5.4 15-Hydroxy-Prostaglandin Dehydrogenase.

Two types of 15-hydroxy-prostaglandin dehydrogenase (PGDH) are known to exist (Hansen, 1976), and they are distinguished by cosubstrate specificity. Type I PGDH is the NAD^+- dependent form, whereas the cosubstrate for PGDH type II is nicotinamide-adenine dinucleotide phosphate ($NADP^+$). In the human, chorion is the major site of prostaglandin metabolism (Okazaki et al, 1981a) reflecting the higher type I NAD^+-dependent PGDH activity in placenta and chorion compared with amnion and decidua (Schlegel and Greep, 1975). This enzyme catalyses the transformation of prostaglandins into their 15-keto derivatives which represents the first step in their degradation.

The distribution and localisation of PGDH in fetal membranes and placenta have been determined by immunocytochemical studies (Cheung et al, 1990, Sangha et al, 1994). These demonstrated that immunoreactive PGDH was absent from the subepithelial and epithelial layers of the amnion; confined to the trophoblast layer of the chorion; and absent from decidual stromal cells, but present in trophoblast cells that had invaded the

decidua. In the placenta PGDH was localised to intermediate trophoblast and syncytiotrophoblast. In the same experiments labour did not influence PGDH localisation within the placenta or membranes suggesting that changes in PGDH distribution or activity are unlikely to be responsible for the increase in prostaglandin concentrations seen with parturition. This is in keeping with the findings of Cheung and Challis (1989) who demonstrated no change in the metabolism of exogenous prostaglandins by chorion obtained pre- and postlabour.

PGDH is thought to represent a metabolic barrier to the transfer of prostaglandins, allowing only small amounts of unmetabolised, active prostaglandins synthesised by the amnion to pass through the fetal mebranes to the myometrium, where they then exert their uterotonic effect. It has been demonstrated that while there is no difference in the localisation of immunoreactive PGDH between patients delivered by caesarean section or following spontaneous labour at term, there is a significant decrease in immunoreactive PGDH in patients delivered preterm in the absence of infection (Sangha et al, 1994), and the amount of PGDH mRNA in the chorio-decidua is lower in this group of patients. These findings support the concept of prostaglandins escaping metabolism owing to a deficiency of chorionic PGDH, and thus effecting myometrial contractility prematurely. However, it has also been shown that PGDH is distributed heterogeneously in chorion raising the possibility that it does not present a complete barrier to prostaglandin transfer (Cheung et al, 1990).

Concentrations of placental PGDH increase in early pregnancy, with a tenfold increase in activity between 7 and 17 weeks gestation (Keirse et al, 1985). The increase is such that, when expressed per mg protein, PGDH activity has already reached levels seen at term by the end of the first trimester. For obvious reasons it is not possible to sample normal pregnancies in the second half of gestation and therefore any changes occurring in PGDH over this period remain unknown.

Regulation of PGDH.

Possible mechanisms controlling the activity of PGDH have been suggested. There is a certain amount of evidence to support hormonal regulation of the enzyme. In the normal menstrual cycle, prostaglandin metabolism in the endometrium is greatest in the mid-secretory phase of the cycle when progesterone concentrations are at their highest (Casey et al, 1980). Experiments in the pregnant guinea-pig employing the antiprogestin RU 486 have shown that this agent reduces prostaglandin catabolism in the myometrium, membranes and decidua, with a 9-fold reduction in the catabolic activity of the chorion (Kelly and Bukman, 1990). In the human, the administration of RU486 *in vivo* reduces prostaglandin metabolism to some extent in decidual tissue cultured *in vitro* (Norman et al, 1991). Immunocytochemical studies have also confirmed reduced decidual PGDH activity in women treated with RU 486 in early pregnancy (Cheng et al, 1993). Therefore, progesterone may be an important regulator of PGDH, and a reduction in PGDH activity could contribute to the increased sensitivity of the uterus to exogenous prostaglandins which is seen in the clinical situation following priming with antiprogestins (Van Look and Bygdeman, 1989). It has also been suggested that PGDH is under genetic control based on studies of placental PGDH activity in multiple pregnancy (Erwich and Keirse, 1988). In addition, the presence of endogenous inhibitors of PGDH has been indicated by the fact that total enzyme activity increases after the initial step in the purification of PGDH in the placenta (Flower, 1974). Further attempts to characterise these inhibitors have suggested that they may be fatty acids (Mibe et al, 1992).

Transfer of Prostaglandins across fetal membranes.

Whilst it is generally accepted that prostaglandins produced by the placenta and fetal membranes are essential to the events resulting in labour, there is some debate as to the exact source of the prostaglandins that facilitate myometrial contractility.

Prostaglandin E_2 is the major prostaglandin produced during active labour and its site of synthesis is the amnion. In order to effect uterine contractility PGE_2 must be able to cross the chorion, escaping the high metabolic activity of this tissue owing to the presence of PGDH, which would result in the production of inactive metabolites. There has been some disagreement in the literature as to whether or not this is possible. McCoshen et al (1990) demonstrated that addition of PGE_2 to *in vitro* culture of amnion discs resulted in transfer of approximately 25% of the prostaglandin from the fetal to maternal side of the amnion. There was no transfer of PGE_2 across intact membranes (amnion-chorion-decidua). Similarly, Sullivan et al (1993) found that PGE_2 levels on the maternal side of cultured intact membranes were 90% lower than on the maternal side of amnion alone. These authors conclude that the metabolic activity of the chorion prevents transfer of PGE_2 produced by amnion and that these prostaglandins do not directly stimulate myometrial activity.

In contrast, Bennett et al (1990) have demonstrated that PGE_2 and 5-hydroxyeicosatetranoic acid (5-HETE) will cross amnion and intact fetal membranes. Transfer of PGE_2 and 5-HETE occurred at equal rates in both directions across amnion and intact membranes, although the transfer of both agents was slower across intact membranes compared with amnion alone. There was no metabolism of PGE_2 after transfer across amnion alone, and 58% of PGE_2 crossed from the fetal to maternal surface of amnio-chorio-decidua intact. These findings are in keeping with those of Nakla et al (1986), although Bennett et al (1990) were unable to demonstrate any change in the rate of transfer with labour. Both groups suggest that transfer is likely to occur by simple diffusion, because total reactivity passed in either direction, and there was no decrease in the rate of transfer with increasing concentrations of test compounds, supporting a lack of receptor or carrier-mediated mechanism.

Further evidence supporting the hypothesis that prostaglandin transfer can occur across intact fetal membranes is provided Johnston et al (1996). This work has the advantage of being performed *in vivo*, overcoming some of the disadvantages of the *in vitro* model. These authors developed an intra- and extra-amniotic dialysis system within the pregnant sheep uterus. They confirmed that appreciable amounts of PGE_2 can cross intact fetal membranes and that there is a relatively constant rate of transfer, again implying a simple diffusion process. There is therefore a certain amount of evidence to support the transfer of prostaglandins across fetal membranes inspite of the metabolic "barrier" of the chorion.

1.6 Cytokines and Parturition.

Cytokines play a central role in a number of biological mechanisms, and their increasing importance in reproductive processes continues to evolve. Cytokines are soluble glycoproteins which act non enzymatically to regulate cellular function (Nathan and Sporn, 1991), usually in an autocrine or paracrine fashion. They can be produced by many different cell types and are capable of exerting their effects on a variety of tissues and target organs. There is increasing evidence to support the concept of a "cytokine network" through which these agents promote their diverse effects (Mitchell et al, 1993a). Cytokine receptors are present on many cell types and it is through binding to these receptors that cytokines stimulate intracellular events. The interaction of cytokine with its receptor results in configurational changes within the receptor. G proteins present in the cell membrane then activate enzymes on the inner cell membrane interface leading to synthesis of a second messenger which will in turn regulate intracellular function. The second messengers activated by cytokines are cAMP and

the IP_3/DAG pathway discussed previously. Examples of cytokines include the interleukins (numbered 1-12) and tumour necrosis factor α (TNFα), and peptide growth factors such as transforming growth factor (TGF) α and β and epidermal growth factor (EGF).

Chemokines are a group of chemotactic cytokines which have four cysteine residues forming two disulfide bonds. They can be categorised into two families: α chemokines in which the first two cysteines are separated by a single amino acid residue and are termed the C-X-C subfamily, and β chemokines which have no intervening sequence and are termed the C-C subfamily. α chemokines are encoded on human chromosome 4 and examples include interleukin 8 (IL-8) and platelet factor 4 (PF-4). The α chemokines have 30 -50% amino acid sequence homology. Both IL-8 and PF-4 cause neutrophil and lymphocyte infiltration *in vivo*. *In vitro* these agents attract neutrophils, T cells and fibroblasts. α chemokines are produced by a number of cells including monocytes and macrophages and their production is induced by agents such as IL-1, IL-3 and tumor necrosis factor (TNF). β chemokines are found on chromosome 17 and include monocyte inflammatory protein-1α and monocyte chemotactic peptide. The members of this subfamily have structural homology with each other and with the α chemokine family. β chemokines,which are produced largely by T cells, in general attract monocytes, and their production is induced by IL-4, TGFβ and corticosteroids

There is considerable interest in a potential role for cytokines in the regulation of the onset of parturition. This has been stimulated by investigations into the underlying mechanisms of preterm labour, implicating a role for inflammatory cytokines (IL-1, 6, 8 and TNFα) in this process. There is growing evidence for a relationship between intra amniotic infection (defined as the presence of microorganisms in amniotic fluid obtained by transabdominal amniocentesis [Romero et al, 1988]) and preterm labour.

It has been demonstrated that 16% of women in preterm labour have intra amniotic infection (Romero et al, 1988). In addition, women with positive amniotic fluid cultures are more refractory to tocolysis, and this group have an increased incidence of histologically confirmed chorioamnionitis when compared to women who respond to treatment (Hameed et al, 1984).

The final common pathway in the onset of preterm labour appears to be increased prostaglandin production (Liggins et al, 1979; Lundin-Schiller and Mitchell, 1990a). Potential stimulants of prostaglandin production in the face of infection include bacteria and cytokines. The bacteria colonising the amniotic fluid have not been shown to be capable of producing prostaglandins directly themselves. However, some bacteria possess phospholipase A_2 activity (Bejar et al, 1981), and are thus able to increase substrate availability for prostaglandin biosynthesis. Investigation into the effect of bacteria on prostaglandin production by amnion and decidua has shown that there appears to be a biphasic dose-response, with low doses of bacterial products promoting prostaglandin synthesis while high doses are inhibitory (Mitchell et al, 1991a). Potentially pathogenic bacteria such as Escherichia coli, Streptococcus viridans and Bacteroides fragilis will stimulate arachidonic acid metabolism by cultured amnion cells via both the cyclooxygenase and lipoxygenase pathways (Bennett et al, 1987). However, the ratio of cyclooxygenase to lipoxygenase metabolism is increased. Importantly, Lactobacillus, a member of normal vaginal flora, did not stimulate prostaglandin production, and further experiments have confirmed that common genital tract pathogens do not metabolise arachidonic acid to prostaglandins or other eicosanoids (Bennett and Elder, 1992a).

Rather than a direct effect of bacteria themselves, the host response to infection, specifically mediated by cytokines, may be the mechanism of preterm labour. This

theory is supported by the finding that interleukins are present in amniotic fluid, and in greater concentrations in the presence of infection.

Interleukin-1 is present in amniotic fluid from the third trimester (Romero et al, 1989a; Romero et al, 1990a) and has the characteristics of IL-1α rather than IL-1β. In preterm labour associated with infection IL-1 activity in amniotic fluid is significantly greater than in preterm labour either without infection, or responsive to tocolysis, and this activity is more consistent with IL-1β. Il-1β concentrations are also increased in amniotic fluid from labouring women at term compared with non-labouring women. Similarly, IL-6, which is present in amniotic fluid from the second trimester onwards (Romero et al, 1990b), is increased significantly in amniotic fluid from women with intrauterine infection. Again, there is a small but significant increase in amniotic fluid IL-6 concentrations in association with labour at term. IL-8 can be demonstrated in amniotic fluid from a proportion of women from mid-pregnancy onwards (Romero et al, 1991), and it is increased in association with infection driven preterm labour. It is also significantly elevated in amniotic fluid from women in labour at term with negative cultures compared to nonlabouring women. TNFα is not detected in amniotic fluid, in either pregnancy or labour, unless there is intrauterine infection (Casey et al, 1989a; Romero et al, 1989b), when substantial amounts will be found in 20-73% of cases.

1.6.1 Intrauterine Sources of cytokines.

The fetal membranes and decidua produce a number of cytokines (Romero et al, 1989c; Dudley et al, 1992; Casey et al, 1989a) and while decidua was thought to be their major source there is evidence to suggest that chorion may be potentially more

important, based on the finding that basal IL-6 and IL-8 production is greater by chorion than decidua (Mitchell et al, 1993a). However, it is likely that the majority of cytokine production in chorioamnionitis is from infiltrating inflammatory cells rather than from the intrauterine tissues. Following the primary stimulus for cytokine production, which is generally an infectious or immunological challenge, there is potential for a cascade of interactions between cytokines, their receptors, and other cytokines. IL-1 can induce expression of IL-8, IL-6 and TNFα, while TNFα can induce expression of these three interleukins. The fetal membranes, particularly the choriodecidual interface, can therefore contribute to this complex interplay of cytokine production, which are in turn capable of stimulating prostaglandin synthesis as will be discussed below.

IL-1 and TNFα will stimulate prostaglandin production from amnion (Romero et al, 1989d), chorion (Lundin-Schiller and Mitchell, 1991b) and decidua (Mitchell et al, 1990). IL-6 stimulates prostaglandin production by amnion and chorion (Kent et al, 1993), and although decidual prostaglandin production was not stimulated in these experiments, other investigators have demonstrated a stimulatory effect of IL-6 in this tissue (Mitchell et al, 1991b). The concentrations of these cytokines necessary to promote such an effect is similar to their concentrations in the amniotic fluid of women with intrauterine infection.

The mechanisms whereby these cytokines promote prostaglandin production have also been investigated. The stimulatory effect of IL-1β on amnion cell prostaglandin production appears after 2 hours of incubation implying an induction process (Mitchell et al, 1993b). This effect is dependent on new protein synthesis since it is inhibited by coincubation with cycloheximide or actinomycin D. The addition of substrate in the form of arachidonic acid has a synergistic effect, suggesting that IL-1β does not mediate its effect by increasing substrate release from glycerophospholipids. The

finding that cells pretreated with acetylsalycylic acid recover more rapidly when treated with IL-1β compared with control implies that induction of the enzyme cyclooxygenase occurs, and increased COX protein has been confirmed by Western blot analysis (Mitchell et al, 1993b). Subsequent Northern blot analysis has demonstrated the presence of mRNA for the inducible form of the enzyme, COX-2, within amnion. It has also been shown that the mechanisms responsible for increased prostaglandin production by chorion, in response to IL-1β, are in keeping with those described above (Pollard et al, 1993), and that these mechanisms are common to TGFα and EGF stimulated prostaglandin production in this tissue.

1.7 The Cervix and Cervical Ripening.

The cervix has numerous functions which are reflected in its unique structure. These include, in the nonpregnant state, facilitation of sperm transport, prevention of microbial invasion and passage of menses and uterine secretions. In pregnancy, the cervix transforms from a rigid sphincter, acting to maintain the intrauterine contents, to a soft, compliant structure capable of dilating to enable delivery of the fetus. These dramatic changes in cervical structure are the result of active metabolic processes occurring within this organ, which are described clinically by the modified Bishop score (Table 1.1. Calder et al, 1974), an adaptation of Bishop's original pelvic score (Bishop EH, 1964).

SCORE	**0**	**1**	**2**	**3**
DILATATION	<1 cm	1-2 cm	2-4 cm	>4 cm
LENGTH	>4 cm	2-4 cm	1-2 cm	<1 cm
CONSISTENCY	firm	average	soft	
POSITION	posterior	mid-anterior		
STATION	0-3	0-2	0-1; 0	+
				TOTAL

Table 1.1. Modified Bishop score (Calder et al, 1974).

The higher the total score, the more ripe the cervix is deemed to be, and therefore the closer the timing of labour onset. This section introduces cervical structure, the physiological changes associated with cervical ripening, and the control thereof.

1.7.1 Cervical Structure.

The endocervical canal is lined by columnar epithelium which contains multiple secretory units. The main body of the cervix is composed of connective tissue, the importance of which in relation to cervical ripening will be outlined in detail below. External to the connective tissue, on the vaginal aspect of the cervix, is a layer of smooth muscle which may have a minor role to play in maintaining cervical closure, but is not considered to be nearly as important as the connective tissue in this mechanism (Danforth, 1960). The outermost layer of the cervix is stratified squamous epithelium.

Before discussing the connective tissue of the cervix specifically, the structure of fibrous connective tissue in general will be briefly outlined. The extracellular matrix of fibrous connective tissue is composed of collagen fibres and elastin separated by ground substance. The function of collagen is to confer tensile strength, whereas elastin gives the tissue elasticity. The exact function of the components of the ground substance is only understood in part.

Collagen.

The basic molecule of collagen is tropocollagen, a protein with a molecular weight of 300,000 daltons. Tropocollagen itself is composed of three parallel polypeptide chains wrapped around each other in a helical structure. Each polypeptide chain has a short nonhelical telopeptide at either end. Apart from the telopeptides, every third residue in the polypeptide chain is glycine and the concentration of the two uncommon amino acids, hydroxyproline and hydroxylysine, is high. The triple helices are held together

by cross links which determine the strength of the collagen fibril. (Fig. 1.5). The number of cross links vary from tissue to tissue and are higher in older collagen.

Elastin.

Elastin provides tissue with the ability to stretch in length by several fold and then return to its original length when the tension is released. It is found in most connective tissues in association with collagen and proteoglycans (Leppert et al, 1982).

Proteoglycans.

Proteoglycans are the largest molecules in the human body with molecular weights of up to 3 $x10^6$ Daltons. They are composed of glycosaminoglycans (previously termed acid mucopolysaccharides) connected to a protein core (Fig. 1.5) The glycosaminoglycans (GAGs) are long chains of highly negatively charged repeating disaccharides containing one hexosamine (glucosamine or galactosamine) and one uronic acid (glucuronic or iduronic). Examples of GAGs include heparin, heparan sulphate, dermatan and chondroitin sulphate, each one varying in composition according to the exact combination of hexosamine and uronic acid residues.

Proteoglycans can be defined according to their protein core and classified by their glycosaminoglycan side chains (Ruoslahti, 1988). Decorin is the predominant proteoglycan in the cervix and consists of a protein core with a single dermatan sulphate chain. Biglycan carries two dermatan sulphate chains which are structurally similar to that found in decorin. A larger proteoglycan, PG-L, contains chondroitin and dermatan sulphate side chains (Uldberg and Malmstrom, 1991).

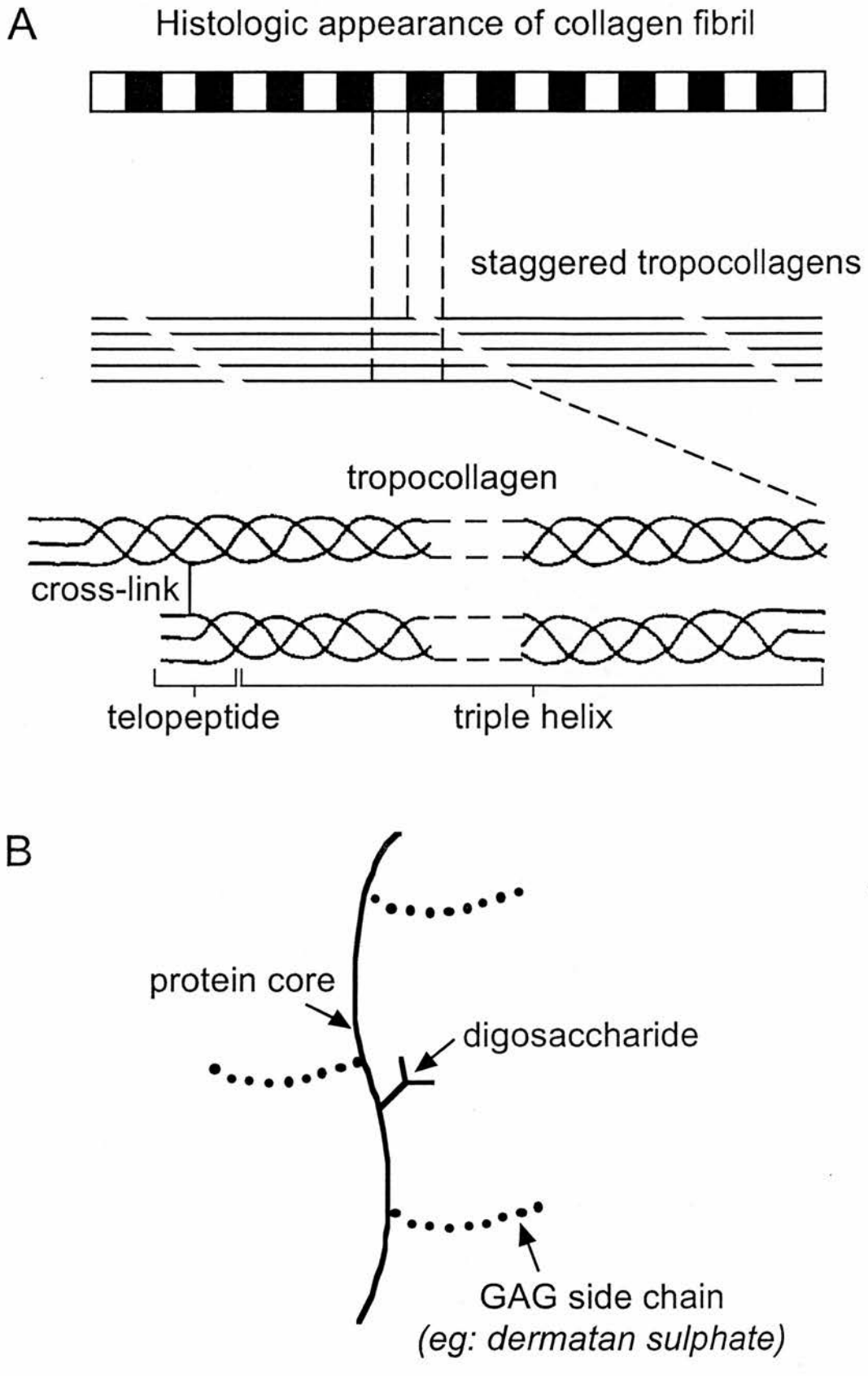


Figure 1.5
Basic structure of collagen (A) and proteoglycan (B).

Proteoglycans are able to attach to collagen via their protein cores and thus invest the collagen fibrils. The GAG side chains can then interact with other collagen molecules, and with each other, thereby influencing the mechanical strength of the connective tissue (Lindahl and Hook, 1978; Liggins, 1978). GAGs bind to collagen with increasing affinity as their chain length and charge density increase, and therefore it can be seen that the proteoglycan/GAG composition is important in influencing the mechanical properties of connective tissue.

Collagen Degradation.

The enzymes collagenase and elastase are capable of collagen degradation; the former is considered essential to this process whereas the latter may be involved mainly in collagen degradation in granulocyte-dependent inflammatory reactions. Collagenase is a member of the matrix metalloproteinase (MMP) family. These zinc-dependent proteolytic enzymes function extracellularly at neutral pH, and they are essential to the degradation and remodelling of the extracellular matrix. MMPs are produced as proenzymes which are activated in the extracellular matrix by proteolytic cleavage. Removal of the "pro" domain of proMMPs results in active enzyme and this cleavage is performed by other MMPs (Hulboy et al, 1997). The MMPs can be classified in to three groups based on their substrate specificity. One of these groups, the interstial collagenases, includes two distinct enzymes: one produced by fibroblasts (MMP-1) and one produced by polymorphonuclear leukocytes (MMP-8). MMP-1 and -8 degrade types I, II, III, VII and X collagen. Production of MMPs is tightly regulated by cytokines, hormones and growth factors, as is the synthesis of their specific inhibitors - the tissue inhibitors of metalloproteinases (TIMPs) (Murphy and Reynolds, 1993).

Secretion by fibroblasts is limited by the rate of enzyme synthesis since these cells do not contain an intracellular pool. Leukocytes, however, store collagenase in their granules and are therefore capable of immediate enzyme release which may account for the rapid degradation of collagen seen in inflammatory reactions. Leukocyte elastase, found in the azurophil granules of leukocytes, degrades elastin, collagen, fibrin, fibrinogen and cartilage proteoglycans. Its activity with regard to collagen degradation is thought to be different from collagenase but it has been suggested that the two enzymes work synergistically.

1.7.2 Physiology of Cervical Ripening.

The cervical stroma is mainly composed of connective tissue containing collagen, elastin and ground substance (proteoglycans), which constitute more than 70% of its dry weight. The predominant types of collagen found in the cervix are type I (66%) and type III (33%) (Kleissl et al, 1978). Microscopic examination of the nonpregnant or unripe pregnant human cervix reveals densely packed collagen bundles, composed of parallel collagen fibrils, which run in different directions. Cells are interspersed betwen this network of which the predominant cell type is the fibroblast. As pregnancy advances and the cervix ripens the histological appearance of the cervical stroma changes. There is a marked increase in vascularity, along with activation of the fibroblasts; findings which are comparable to those seen in inflammatory reactions and wound healing (Liggins 1981). This increase in cellular activity results in dispersal of the compact collagen bundles such that they appear scattered and partly dissolved on microscopic examination.

A number of changes in the biochemical properties of the cervix are known to occur in association with pregnancy, and are thought to be central to the process of cervical ripening which facilitates parturition. These include a reduction in cervical collagen concentration, an increase in water content and a change in the proteoglycan/GAG content of the tissue. These changes will now be discussed in detail.

Collagen.

Measurement of hydroxyproline concentrations in cervical biopsies obtained in the non pregnant state and at various stages of pregnancy have established that collagen concentration decreases by approximately 70% during pregnancy (Uldbjerg et al, 1983a). There is already a significant fall in collagen concentration by 10 weeks gestation which is in keeping with the clinical finding of cervical softening that was once used to diagnose pregnancy. There is a further significant fall in collagen concentration as gestation progresses to term, but interestingly there is no further change with labour.

The physicochemical properties of cervical collagen are also altered in relation to gestation such that the extractability of collagen in acetic acid with pepsin is much higher in the pregnant compared with the nonpregnant state and increases significantly during labour. These findings are in keeping with those of other investigators (Ito et al, 1979; von Maillot and Zimmermann, 1976). It has been demonstrated that the relative amount of partially degraded collagen is higher in cervical biopsies collected at term or immediately postpartum compared with those obtained from nonpregnant women (Kleissl et al, 1978). In addition, the number of intermolecular cross links is reduced in term and postpartum specimens. This suggests that mature collagen, which is relatively insoluble, is being broken down and replaced by new collagen with fewer

cross links which is soluble and therefore easy to extract, and is more readily digested by collagenolytic enzymes.

A proposed explanation for the lack of change in collagen concentration in association with labour itself, inspite of high collagenolytic enzyme activity, is that the relative proportion of old and new collagen has changed. The higher concentration of new collagen is easily digested, but it may be that the degradation products are retained in the tissue for some time (Kleissl et al, 1978) and hence the total collagen concentration is not affected by parturition. This would also explain the increased extractability of collagen in postpartum biopsies. There is also histochemical evidence to support the retention of degradation products within the cervix (Junqueira et al, 1980). It is important to note that there is no change in the relative concentrations of type I and type III collagen in the nonpregnant and pregnant cervix (Huszar et al, 1981). The importance of changes in collagen content in relation to the clinical setting is illustrated by Ekman et al (1986) who demonstrated that women in spontaneous labour with an unfavourable cervix (low cervical score) had a higher collagen concentration than women with a high cervical score.

Collagenase.

Collagenase has been extracted from the human cervix (Kitamura et al, 1980a) and 22% of this enzyme is present in the free, active form. Of the remainder, 69% is complexed to α_2-macroglobulin and 9% exists as proenzyme. Collagenase activity, measured as DNP peptide hydrolytic activity, is significantly increased in early pregnancy and continues to increase through to term (Uldbjerg et al 1983b). Similar changes are also seen in the activity of leukocyte elastase (Uldbjerg et al 1983b). There are contrasting findings with regard to the effect of labour on collagenase activity. Serum collagenase activity is increased once labour is established (Rajabi et al, 1985) and higher collagenase activity has been demonstrated in cervical biopsies

from women in established labour compared with women at term, and not in labour (Osmers et al, 1990; Rajabi et al 1988). However, Uldbjerg et al (1983b) found no increase in collagenase activity during labour as did Granstrom et al (1989).

Proteoglycans

Dermatan and chondroitin sulphate are the predominant proteoglycans in the human cervix (Uldbjerg et al, 1983a; von Maillot et al, 1979). Dermatan sulphate is a relatively small proteoglycan with a molecular weight of 100,000 daltons. These molecules are responsible for promoting tensile strength in the cervix. Indeed it has been suggested that tissue rigidity is proportional to chondroitin sulphate concentration (Bryant et al, 1968), and therefore a reduction in chondroitin sulphate could improve cervical compliance. The total GAG content of the cervix increases during pregnancy, indicating that active synthesis is taking place, however the actual concentration of GAGs remains relatively unchanged (Golichowski, 1980). As alluded to previously, a change in the relative concentrations of different GAGs could affect collagen binding and hence tissue stability.

The concentration of sulphated glycosaminoglycans is known to be lower in cervical biopsies obtained postpartum compared with those obtained from nonpregnant women (Kitamura et al, 1980b; Cabrol et al, 1980). This reduction in sulphated GAG concentration is not as marked as the decrease in collagen concentration associated with pregnancy, and may be explained by an opposing increase in heparan sulphate concentration, found in blood vessels within the tissue (Kitamura et al, 1980b).

Some studies have demonstrated an increase in hyaluronic acid with advancing gestation (von Maillot et al, 1979; Golichowski, 1980). Hyaluronic acid can exist as a free GAG, unbound to proteoglycan, and because it binds least strongly of the GAG molecules it will act to destabilise collagen. In addition, because of its highly

hydrophilic properties, an increase in hyaluronic acid concentration can lead to increased tissue hydration resulting in increased cervical compliance. This could explain the change in water content of the cervix that occurs with pregnancy, increasing from 80% in the nonpregnant state (Liggins, 1978) to 86% in late pregnancy (Uldbjerg et al, 1983a). However, a number of studies have not confirmed the increase in hyaluronic acid concentration (Uldbjerg et al, 1983a; Uldbjerg and Malmstrom, 1991) and therefore other biochemical changes within the cervix may be important.

1.7.3 Control of cervical ripening.

The above evidence illustrates that synthesis as well as catabolism is occurring throughout pregnancy, and that continuous remodelling is taking place within the cervix. Cervical ripening is therefore likely to be an active process rather than a passive one reliant on uterine contractility, as was once thought. The fact that physical isolation of the cervix from the uterus, as demonstrated in animal studies (Ledger et al, 1985), does not prevent the ripening process lends further support to this theory. A number of agents, including prostaglandins, oestrogen, progesterone and cytokines, are thought to be involved in the regulation of cervical ripening and will be be discussed in further detail.

Prostaglandins

Prostaglandins have an established role in the pharmacological ripening of the human cervix and are able to ripen the cervix at all stages of pregnancy (Calder, 1979; Calder and Greer, 1991). The cervix can synthesise prostaglandins and the main ones

produced are PGE_2 and prostacyclin (PGI_2), with production of $PGF_{2\alpha}$ occurring in smaller amounts. Cervical prostaglandin production increases at term (Ellwood, 1979), suggesting that they have a physiological role in the ripening process. In addition it has been demonstrated that amniotic fluid prostaglandin concentrations correlate directly with cervical score in women at term but not in labour (Calder and Greer, 1991). Finally, receptors for PGE_2 and $PGF_{2\alpha}$ can also be demonstrated in the cervix (Crankshaw et al, 1979).

PGE_2 treatment does lead to a reduction in collagen concentration and promotes changes similar to those seen in physiological ripening (Ekman et al, 1986; Uldbjerg et al, 1981). The exact mechanism responsible for this finding is not entirely clear since studies investigating the effect of prostaglandin treatment on collagenase activity have produced contrasting results. Some have reported an increase in collagenase or collagenase-like hydrolytic activity following PGE_2 administration (Ding et al, 1990), whereas others have found no change, or a reduction in enzyme activity in association with treatment (Uldbjerg et al 1983c; Rath et al, 1987). Indeed, in addition to demonstrating no change in collagenase activity with prostaglandin analogue treatment, Rath et al (1987) also found an absence of collagen degradation products on electrophoresis of tissue biopies from the treatment group. These findings suggest that prostaglandins may have no direct effect on collagenase activity *in vivo*. Similarly, *in vitro* experiments have shown that PGE_2 and $PGF_{2\alpha}$ have no effect on collagen breakdown (Hillier and Wallis, 1981).

Arachidonic acid, on the other hand, will increase collagen breakdown, an effect which is blocked by phospholipase inhibitors but not cyclooxygenase inhibitors (Hillier and Wallis, 1981). The step in the arachidonic acid cascade at which the inhibition of the arachidonic acid induced collagen breakdown occurs implies that prostaglandins are not the mediators of this effect. Cervical tissue treated with

prostaglandins or arachidonic acid produces increased amounts of unidentified arachidonic acid products which are not prostaglandins (Christensen and Bygdeman, 1985). It may be that the ripening effect of arachidonic acid is a result of leukotriene production, which can also be stimulated by PGE_2. A potential role for leukotrienes in the cervical ripening process is in keeping with the hypothesis that this may be an inflammatory mediated event.

As discussed above, changes in the proteoglycan/GAG composition of the cervical ground substance will act to destabilise the collagen fibrils thus increasing cervical compliance, and provides another potential mechanism for prostaglandins to produce their effect. PGE_2 can influence cervical fibroblast production of collagen and GAG such that when collagen synthesis is reduced GAG production is increased (Norstrom, 1984). The increased GAG concentrations seen in response to prostaglandin treatment may be the result of increased synthesis, perhaps mediated via induction of hyaluronic acid synthetase within fibroblasts (Murota et al, 1977), or it may be the result of increased breakdown of proteoglycan complexes leading to increased free GAG molecules.

This could result in increased hyaluronic acid concentrations and hence tissue hydration which has been demonstrated in animal models (Cabrol et al, 1987). However, not all studies have demonstrated an increase in hyaluronic acid concentration in response to prostaglandin treatment (Uldbjerg et al, 1983c). Further evidence to support a role for proteoglycan breakdown or GAG production is provided by the finding that circulating levels of dermatan/chondroitin sulphate are increased in association with PGE_2-induced cervical ripening at term (Greer, 1992), and similar increases are seen with spontaneous labour.

Oestrogen.

In sheep an increase in the plasma oestrogen/progesterone ratio has been clearly demonstrated prior to the onset of parturition. This does not occur in humans, but it has been suggested that local changes in the hormonal milieu may occur in association with labour. There is certainly evidence to indicate that oestrogen and progesterone are involved in the control of cervical ripening in women. Intravenous infusion of 17β-oestradiol will promote cervical ripening at term, confirmed on histological examination (Pinto et al, 1965). Similarly, intravenous injection of dehydroepiandrosterone sulphate at term leads to cervical ripening within hours of treatment. Placental metabolism of DHAS results in increased 17β-oestradiol concentrations in both plasma and cervical tissue, and a four-fold increase in cervical collagenolytic activity (Mochizuki et al, 1978). DHAS treatment in rats is associated with an increase in active collagenase concentrations and a decrease in collagenase complexed with α_2-macroglobulin (Kitamura et al, 1981).

Oestradiol has been shown to be an effective cervical ripening agent in the clinical situation when applied topically (Gordon and Calder, 1977). This effect may be due in part to stimulation of prostaglandin synthesis (MacKenzie, 1981). It has been suggested that oestrogen sensitises the cervix to prostaglandins which would explain the increase in pharmacological potency of prostaglandins as gestation advances. Indeed, it has been demonstrated that induction of labour with prostaglandins is easier to accomplish in women with high serum oestradiol concentrations (MacKenzie et al, 1979).

Progesterone.

Animal and clinical studies indicate that progesterone is important in the control of cervical ripening. These studies have focused on the use of antiprogestins such as

onapristone and mifepristone (RU 486) for cervical priming and labour induction. In nonpregnant guinea-pigs cyclical changes occur in the extensibility and dilatation of the cervix which are inversely related to serum progesterone concentrations (Chwalisz et al, 1991). In pregnant guinea-pigs treated with onapristone there is dramatic softening and dilatation of the uterine cervix, prior to the onset of labour. This is reflected histologically by dissolution and dissociation of the collagen fibres due to oedema, and an inflammatory infiltration of polymorphonuclear granulocytes, macrophages and mast cells (Hegele-Hartung et al, 1989). It is interesting that both onapristone and RU 486 will induce similar changes in the nonpregnant cervix, so that pregnancy is not essential for this response (Chwalisz et al, 1991). Administration of a pure progesterone agonist, promegestone, will completely reverse the effects of onapristone on the cervix, indicating that onapristone exerts its effect via the progesterone receptor.

Onapristone treatment results in a decrease in cervical collagen and glycosaminoglycan concentrations in the guinea-pig in late pregnancy (Chwalisz et al, 1991), indicating that collagenase-mediated collagenolysis causes cervical ripening in guinea-pigs. It has also been demonstrated that progesterone will inhibit expression of the procollagenase gene (Rajabi et al, 1991), and will block increased collagenase production stimulated by oestradiol. Therefore, progesterone may act directly or indirectly to control collagenase activity within the cervix.

Mechanisms which could be responsible for the cervical ripening effect of antiprogestins have been investigated. It appears that prostaglandins do not mediate the effect of onapristone and RU 486 in promoting cervical ripening. This is based on the findings that indomethacin does not inhibit onapristone-induced cervical ripening in guinea-pigs (Chwalisz et al, 1991), and that naproxen, another prostaglandin synthase inhibitor, will not inhibit the effect of RU 486 on the human cervix (Radestad and Bygdeman, 1992). As discussed above, onapristone treatment results in an

inflammatory cell infiltrate suggesting that chemotactic agents such as cytokines may mediate the effects of antiprogestin treatment, and further evidence for this will be presented below.

Relaxin.

Relaxin was discovered by Hisaw (1926) who found that injection of serum obtained from pregnant guinea-pigs into virgin guinea-pigs shortly after oestrus caused relaxation of the pelvic ligaments. The changes in the connective tissue of the pubic ligaments in these animals involve dissociation of the collagen bundles, a net increase in total collagen, increased water uptake and a decrease in the viscosity of the ground substance. Animal studies (Steinetz, 1980) suggest that relaxin-induced cervical ripening is similar to that seen prior to parturition and that the connective tissue changes are similar to those seen in the pubic symphysis described above. *In vitro* studies on the rat pubic symphysis have demonstrated that relaxin increases tissue levels of collagenase and collagen peptidase (Weiss et al, 1979), implying stimulation of the collagenolytic system. Relaxin also has mitogenic properties which appear to stimulate the replication and synthesis of new collagen. Thus relaxin appears to promote remodelling of the target tissue by influencing both collagen synthesis and metabolism. In addition, by increasing the water and glycosaminoglycan content of the tissue, relaxin also decreases the viscosity of the intercellular ground substance (Zarrow et al , 1956). The net result is a relative decrease in the collagen concentration of the target tissue with a resultant increase in tissue distensibility.

Oestrogen priming is a prerequisite for relaxin's effect on the guinea-pig pubic symphysis (Hisaw, 1926), and inhibition of steroid synthesis in the pregnant rat will block relaxin's effect on the cervix (Schwabe et al, 1978). Further support for a role for steroid hormones in the control of relaxin physiology comes from studies

demonstrating that oestrogen may affect the release of relaxin in some animals, and that oestrogen may stimulate an increase in relaxin receptor sites (Mercado-Simmen et al, 1980). Prostaglandins are also implicated in relaxin's effect on cervical ripening, since administration of prostaglandin synthetase inhibitors will block the cervical ripening effect of relaxin in rats (Kennedy, 1976).

Because relaxin does not stimulate myometrial contractility there has been a lot of interest in relaxin as a potentially ideal cervical ripening agent. Purified porcine relaxin has been shown to be effective as a cervical ripening agent in humans (MacLennan et al, 1980; Evans et al, 1983). This is inspite of the lack of structural homology between porcine and human relaxin. The development of recombinant human relaxin has stimulated investigation into its efficacy as a cervical ripening agent and one such trial is discussed in chapter six.

Cytokines.

The mechanisms resulting in cervical ripening have been likened to that of an inflammatory process (Liggins, 1981). There is an infiltration of leukocytes into the human cervix at term and it has been proposed that these cells are responsible for triggering the connective tissue changes that take place during the ripening process (Junqueira et al, 1980; Osmers et al, 1992). The neutrophil is the predominant white blood cell found in the cervix during normal parturition and since these cells, together with macrophages, are a source of collagenases (Ito et al, 1987) they may have an important role in cervical ripening. Investigation into cervical ripening in the guinea-pig has demonstrated that inflammatory cells infiltrate the cervix during spontaneous ripening at term (Hegele-Hartung et al, 1989). This influx of inflammatory cells may be under the control of progesterone since treatment with the progesterone antagonist onapristone will increase the cellular infiltration (Chwalisz et al, 1991).

Cervical explants from pregnant rats (Ito et al, 1988) and pregnant women (Barclay et al, 1993) are capable of producing IL-1 and IL-8 [(neutrophil attractant/activating peptide-1 (NAP-1)]. As its name suggests, IL-8 is a potent neutrophil chemotactic agent and promotes the release of collagenolytic enzymes from these cells (Peveri et al, 1988). Because of the influx of neutrophils into the cervix previously discussed, and the fact that IL-8 concentrations in amniotic fluid are increased in association with spontaneous labour (Romero et al, 1991) it is not unreasonable to hypothesise that IL-8 may play a significant role in the onset of parturition at term. It has been shown that topical application of IL-8 and IL-1β can promote cervical ripening in guinea-pigs (Chwalisz et al, 1994) and that the associated morphological changes are similar to those induced by antiprogestational agents (Hegele-Hartung et al, 1989). In this study (Chwalisz et al, 1994), treatment did not lead to premature labour implying that the cytokines are exerting a local effect rather than increasing myometrial contractility.

There is evidence to support a role for progesterone in the regulation of cytokine production in various uterine tissues. The IL-8 gene contains a glucocorticoid/progesterone response element, so that IL-8 may be directly down-regulated by progesterone (Matsushima and Oppenheim, 1989). Progesterone will decrease IL-8 production by choriodecidual cells *in vitro*, whereas RU 486 has the opposite effect (Kelly et al, 1992). A similar effect on IL-8 production is seen following progesterone treatment of endometrial explants (Kelly et al, 1994), and progesterone withdrawal will stimulate neutrophil infiltration into the endometrium in pregnant sheep (Staples et al, 1983), and into the placenta in pregnant monkeys (Sinosich et al, 1989), implying a cytokine-mediated effect. Thus progesterone may be acting as an immunosuppressor within the cervix and antiprogestational treatment or spontaneous progesterone withdrawal may activate the cytokine cascade and hence cervical ripening (Chwalisz et al, 1994). However, treatment with the antiprogestin RU 486 does not promote cervical ripening in all species. In contrast, RU 486 will

stimulate uterine activity to the extent of fetal hypoxia in the monkey without promoting cervical ripening, whereas administration of the 3β hydroxysteroid dehydrogenase inhibitor epostane will successfully achieve ripening in this species (Haluska and Novy, 1993). These data suggest that if progesterone withdrawal is important in parturition it may not be a receptor-mediated effect.

1.8 Control of Parturition

1.8.1 Ovine Model.

The mechanisms of parturition have been investigated extensively in the sheep and whilst these cannot be applied directly to the situation in humans, certain similarities exist. It is therefore worth illustrating the events which culminate in labour in this species.

It is established that the fetus plays a central role in orchestrating the timing of parturition in the sheep. Liggins (1973) demonstrated that fetal hypophysectomy or bilateral fetal adrenalectomy will result in prolongation of pregnancy. In contrast, infusion of adrenocorticotrophin (ACTH) or administration of cortisol to the fetal lamb *in utero* provoked the onset of labour within a consistent time interval. These findings are peculiar to the fetus since administration of ACTH or cortisol to the mother, or maternal hypophysectomy, at the same gestation did not influence the timing of labour.

There is a large body of evidence to support maturation of the hypothalamo-pituitary-adrenal (HPA) axis in the fetus prior to the onset of labour. During the final 20 to 25 days of gestation there is an increase in fetal plasma cortisol concentrations (Bassett and Thorburn, 1969) which reach a maximum two to three days prior to delivery. This is likely to represent an increase in cortisol secretion, and during late gestation the adrenal gland is known to undergo both hypertrophy and hyperplasia. There is no change in maternal cortisol levels over the same time period, indicating that the increased fetal concentrations are not simply the result of increased maternal transfer. These changes in cortisol are dependent on the fetal pituitary, since maternal ACTH does not cross the placenta (Jones et al, 1975) and fetal hypophysectomy results in hypocortisolaemia (Kendall et al, 1977). Radioimmunoassay techniques for ACTH have demonstrated a progressive increase in ACTH concentration during the last 30 days of gestation, prior to the increase in cortisol levels (Wintour, 1984).

The pituitary releases ACTH in response to corticotrophin releasing factor (CRF). In the fetal sheep, injection of synthetic ovine CRF will stimulate ACTH release, but the magnitude of this response depends on gestational age: a greater response is seen in the later stages of pregnancy (Challis and Olson 1988). The same was true for cortisol release by the adrenal gland in these experiments. These findings support a maturational process within the HPA axis as gestation advances, with increasing sensitivity of both the pituitary and adrenal gland. Argenine vasopressin (AVP) is another corticotrophic agent, and has been shown to act synergistically with CRF in stimulating ACTH release (Durand et al, 1986). Other factors important in controlling ACTH release may include endogenous opioids since administration of the opioid antagonist naloxone will decrease circulating concentrations of ACTH in the fetal sheep (Challis et al, 1991). Additionally, it has been shown that mRNA for the precursor peptide of ACTH, pro-opiomelanocortin (POMC), increases from day 125

of gestation to term (Challis et al, 1991) suggesting increased POMC gene transcription which may contribute to the increase in ACTH prior to labour.

Prostaglandins also make an important contribution to the regulation of ACTH and cortisol release. During late pregnancy the activity of COX increases in the sheep placenta to reach a maximum at term (Thorburn and Rice, 1990). It has been proposed that the placenta is an important source of trophic stimulus for maturation of the HPA axis, and that the increasing amounts of PGE_2 produced in late pregnancy could be potentially important (Thorburn and Rice, 1990). A correlation between the rise in fetal plasma cortisol and PGE_2 concentrations has been established in late pregnancy (Thorburn and Rice, 1990), and it is also known that exogenous PGE_2 will stimulate ACTH and cortisol production.

The importance of cortisol in the process of parturition is that it promotes transcription of the p450 17α gene which leads to an increase in placental 17α hydroxylase activity (Liggins, 1973). Since progesterone is a poor substrate for placental 17α hydroxylase in the sheep, metabolism is diverted via pregnenolone to 17-hydroxy pregnenolone and then dehydroepiandrosterone (DHAS). DHAS can then be converted to oestrogens by the action of placental aromatases, the activity of which is also increased in the presence of cortisol. These changes in the metabolism of the placental steroids culminate in an increase in the oestrogen:progesterone ratio, and the progesterone withdrawal characteristic of sheep parturition.

Cortisol is therefore central to the mechanisms of parturition in the sheep. It exerts a number of feedforward effects that ensure continuing or increased cortisol secretion within the fetal unit. It is stimulatory to the adrenal gland itself, perhaps by increasing ACTH receptors (Challis and Roberts, 1988). It also promotes production of corticosteroid binding globulin (CBG) (Challis et al, 1985), which by binding cortisol

can maintain low free cortisol concentrations in the fetus and therefore allow continued CRF and ACTH release inspite of increasing total cortisol concentrations .

1.8.2 Human Model.

While there are many similarities between ovine and human parturition evidence supporting a major role for the fetus in the onset of parturition in the human is not as convincing as in the sheep. Examination of data from a series of 147 anencephalic pregnancies (Swaab et al, 1976) failed to demonstrate any difference in the gestational length of these pregnancies compared with a control population. However, in the anencephalic group, and in a subsequent study looking at experimental anencephaly in monkeys (Novy et al, 1977), there was a much wider variation around the mean gestational age at delivery suggesting that the fetus does play a more minor role in the fine-tuning of gestational length. Clearly, the role of the fetus cannot be investigated to the same extent in the human as in the sheep, making it necessary to postulate the best hypothesis to draw the pieces of the jigsaw together. Some of the concepts of parturition in the human will be discussed below.

The major difference between humans and sheep is that there is no evidence for systemic progesterone withdrawal prior to the onset of labour in humans (Thorburn and Challis, 1979). Extensive investigation of maternal plasma concentrations of total and non-protein-bound progesterone and oestradiol have failed to show any difference in the relative amounts of these hormones prior to the onset of labour (Anderson et al, 1985). However, events occurring locally within the reproductive tissues may not necessarily be reflected in changes in systemic hormone concentrations, and regulation of steroid hormone metabolism by the fetal membranes and decidua may be important

in determining paracrine events. Amnion, chorion and decidua can synthesise oestrogen and progesterone, and changes in the synthesis and metabolism of these hormones may be important in effecting local progesterone withdrawal. Oestrone is the major oestrogen formed by fetal membranes, although its production is not altered by labour (Romano et al, 1986). However, in association with labour, the enzyme 17β, 20α hydroxysteroid dehydrogenase promotes conversion of oestrone to oestradiol, a more active oestrogen. In addition this enzyme enhances the formation of inactive progesterone metabolites thus establishing a significant increase in the oestrogen: progesterone ratio within chorion and amnion (Mitchell and Wong, 1993). It has also been demonstrated that oestrogen and progesterone receptors are present in fetal membranes and decidua and that the concentration of mRNA for the oestrogen receptor was increased three-fold in association with labour in these experiments. There is therefore the facility for changes in hormone concentrations and receptor numbers within the fetal membranes.

Amnion, chorion and decidua possess 3β hydroxysteroid dehydrogenase activity (3βHSD) (Gibb et al, 1978) which is the enzyme responsible for converting pregnenolone to progesterone. Inhibition of this enzyme, which occurs in the presence of oestrogen (Mitchell et al, 1982), would result in a decrease in progesterone production and effect progesterone withdrawal locally. Maternal systemic oestrogen concentrations increase progressively in the last 5-6 weeks of pregnancy (Tulchinsky et al, 1972), and there is also a rapid rise in amniotic fluid concentrations in the 15-20 days leading up to parturition (Turnbull et al, 1977), which could in theory modulate 3βHSD activity.

Although the onset of labour in the human cannot be directly attributed to fetal control, interactions between the fetus and placenta are clearly important in the regulation of this process. Maternal plasma concentrations of CRF rise throughout gestation and

decline rapidly following delivery (Campbell et al, 1987; Goland et al, 1986). This phenomenon is also known to occur precociously in cases of preterm labour (Campbell et al, 1987). The placenta is the source of this CRF and placental content of CRF increases progressively throughout pregnancy to a maximum at term. This is paralleled by an increase in prepro CRF mRNA, demonstrating placental CRF gene expression (Frim et al, 1988). The syncytiotrophoblast is the site of origin of CRF (Riley et al, 1991), with no change in immunostaining for CRF in placental tissue collected prior to or post labour onset. Amnion, chorion and decidua also produce CRF (Jones et al, 1989), with increased production from tissue collected following spontaneous labour. The CRF produced in these experiments subsequently stimulated ACTH release from ovine pituitary cells. It has been suggested that placental CRF stimulates fetal ACTH production thus initiating important events in the fetal pituitary-adrenal axis. Umbilical venous plasma has a higher CRF content than arterial plasma indicating that secretion into the fetal circulation does occur (Goland et al, 1986).

CRF production by placental and fetal membrane cell cultures can be influenced by a variety of agents. It has been demonstrated, *in vitro*, that progesterone will decrease CRF production by amnion cells in early pregnancy (Jones and Challis, 1990) and by amnion and placental cells at term (Jones et al, 1989). This leads to the hypothesis that progesterone withdrawal, either physiological, or pharmacologically mediated, could result in increased CRF production and hence maturation of the HPA axis. In addition, CRF will stimulate PGE_2 and $PGF_{2\alpha}$ production by amnion, decidua and placental cell culture (Jones and Challis, 1990), and such prostaglandins are capable of facilitating cervical ripening and myometrial contractility. Prostaglandins, in addition to cytokines and catecholamines, can stimulate CRF production (Riley and Challis, 1991), establishing a positive feedforward loop. CRF is itself able to stimulate myometrial contractility *in vitro* (Quartero and Fry, 1989), an effect which is inhibited by indomethacin, implying that it may be mediated in part by prostaglandins.

However, Cooper et al (1994), investigating placental CRF immunostaining during pregnancy and labour, found no evidence for regulation of CRF production by either mifepristone or prostaglandins. The lack of effect of prostaglandins may not be surprising given the short period of administration prior to termination of pregnancy (TOP), but mifepristone was administered 24 hours prior to TOP and therefore any effect of this treatment should have been apparent. The authors suggest that the lack of effect of mifepristone may be gestationally related, since Jones et al (1989) found that progesterone inhibited CRF release by placenta at term, but not in early pregnancy (Jones and Challis, 1990). Also, glucocorticoids have been shown to stimulate CRF production (Jones et al, 1989) and prepro-CRF mRNA expression by placental tissue (Robinson et al, 1988), and since mifepristone has additional antiglucocorticoid effects, these may be masking its antigestagenic actions to some extent.

Unlike the sheep model, fetal cortisol does not promote events which lead to an increase in the oestrogen:progesterone ratio. This is because 17α hydroxylase is absent from human placenta. However, DHAS production may be of significance since placental aromatases can metabolise it to oestrogens, thus increasing local concentrations of oestrogen which as discussed earlier is important in promoting synthesis of stimulatory prostaglandins, oxytocin receptors and gap junctions. Placentally derived CRF can stimulate fetal pituitary ACTH production with a resultant increase in adrenal production of hormones such as cortisol and DHAS. Cortisol is paradoxically able to stimulate further placental CRF production thus establishing a positive feedforward cascade loop.

1.9 Overview and Aims of Thesis

Our current knowledge dose not permit a complete understanding of the mechanisms controlling the onset of parturition in humans. The feto-placental unit is likely to be central to this control but evidence available to date suggests that the fetus plays a more minor role in orchestrating events compared with other species. Whilst there is no evidence for systemic progesterone withdrawal local events within the uterus may be important in influencing the hormonal milieu resulting in an increase in oestrogen: progesterone ratio. Oestrogen can then act in a paracrine manner to promote gap junction formation, oxytocin receptor expression and prostaglandin production within the uterine compartment to favour myometrial contractility.

Prostaglandins are important mediators of uterine contractility and are thought by many to be critical to the labour process. The fetal membranes are an important site of prostaglandin synthesis and metabolism, and either an increase in synthesis or a decrease in metabolism could lead to the increase in prostaglandin concentrations associated with labour. Prostaglandin synthesis and metabolism may be influenced in a paracrine fashion by changes in the intrauterine hormonal milieu, and / or by factors present for example in amniotic fluid, which may be under fetal control. There is a strong body of evidence to support a role for cytokines in the process of labour, and it is likely that labour and cervical ripening are inflammatory-mediated events. Cytokines such as IL-1, IL-6 and IL-8 are increased in amniotic fluid in association with spontaneous labour at term, and these inflammatory agents can stimulate prostaglandin production by fetal membranes and may also mediate the histological changes seen in cervical ripening.

Experiments employing progesterone receptor antagonists indicate that progesterone does play an important role in human parturition. There is certainly evidence to

suggest that progesterone exerts an immunosuppressive effect with regard to cytokine production within the uterus since it will inhibit chorio-decidual and endometrial interleukin-8 production. Progesterone withdrawal could therefore facilitate inflammatory events mediated by cytokines which are central to cervical ripening and prostaglandin production within the uterus. In addition progesterone withdrawal will increase myometrial sensitivity to exogenous oxytocic agents thereby having a preparatory effect on the uterus.

Whilst it is likely that cervical ripening is an inflammatory process, the role of a number of hormonal agents in pharmacological ripening has been investigated. Relaxin will promote connective tissue changes in oestrogen-primed animals without stimulating uterine activity. In humans porcine relaxin has been effective in promoting cervical ripening and this may in fact have been an inflammatory-driven process owing to impurities of the agent. Alternatively, local increases in oestrogen: progesterone ratio may be important in relaxin's ripening effect.

We hypothesise that parturition is ultimately an inflammatory-mediated process which facilitates prostaglandin production and cervical ripening resulting in coordination of myometrial contractility and dilatation of the cervix. The exact trigger for the onset of labour is unknown but is likely to involve local changes in oestrogen: progesterone ratio. Such changes may promote cytokine production which in turn leads to increased prostaglandin production by fetal membranes and myometrial activity. Within the cervix cytokines promote connective tissue changes leading to cervical ripening. Changes in steroid hormone concentrations also have a preparatory effect on the uterus increasing myometrial sensitivity to the oxytocic agents liberated as a result of inflammatory mediators. Changes in the oestrogen: progesterone ratio may also have a preparatory effect on the cervix to facilitate the action of other hormones in pharmacological cervical ripening.

The aims of this thesis were:

1. To investigate, in parallel, the synthesis and metabolism of prostaglandins in explants of amnion and chorion respectively to see if alteration in either or both of these processes is responsible for the increase in prostaglandin concentrations seen in association with labour. Also, to determine any effect of steroid hormones and RU 486 on prostaglandin metabolism by chorion.

2. To investigate the effect of amniotic fluid on prostaglandin production by amnion and chorion cell cultures and to establish any effect of amniotic fluid on prostaglandin metabolism by chorion.

3. To investigate mechanisms, which could potentially be promoted by cytokines, by which amniotic fluid may stimulate prostaglandin production by amnion and chorion cell cultures by employing inhibitors of translation, transcription, protein kinase C and tyrosine kinase.

4. To investigate the role of recombinant human relaxin and the progesterone antagonist mifepristone (RU 486) in pharmacological cervical ripening in women with an unfavourable cervix prior to induction of labour at term.

Chapter Two

Methodology

2.1 Introduction to Tissue Culture.

Tissue culture was first described at the beginning of this century (Harrison, 1907) . Harrison chose the frog as his first source of tissue, perhaps because it is a cold-blooded animal and therefore incubation would not be required. Initial cultures used whole tissue, and it is only since the 1950's that there has been expansion into the field of cell culture utilising dispersed cells. The technique of tissue culture has enabled investigation of a number of aspects of cellular function. Intracellular activity such as the replication and transcription of DNA and protein synthesis, and the transfer of RNA, hormones and metabolites within the cell can be studied. In addition, the effect of external factors, for example infection and drugs, on intracellular mechanisms can be explored, as can the interaction of cells with each other.

The advantages of tissue culture are that the physiochemical environment can be precisely controlled and the physiological conditions can be kept relatively constant. Also, cultured cell lines will assume a uniform constitution as the selective pressure of the culture conditions will favour a homogenous culture of the most vigorous cell type. However, there are a number of disadvantages associated with tissue culture which must be recognised. The dissociation of cells and propogation on a two-dimensional substrate will mean that specific cell interactions characteristic of the tissue will be lost. Also, whilst the culture environment can be constantly maintained *in vitro*, certain important homeostatic regulatory mechanisms will not be present and therefore the system may not accurately reflect the *in vivo* situation.

There are three main methods of establishing a culture (Schaeffer, 1979): organ culture suggests that the original architecture of the tissue is maintained; explant culture employs a fragment of tissue, whereas cell culture implies mechanical or enzymatic

dispersal of tissue resulting in a cell suspension. In the primary culture a series of selection processes take place which result in the proliferation of some cells with the demise of other cells that are not suited to the culture conditions. As a result the distribution of cell types will change until all available culture space is occupied (monolayer culture). After confluence is reached (cells are in close contact with each other and all culture space utilised) the proportion of cells that are non-sensitive to density restriction will increase. The work presented in this thesis employs the techniques of explant and cell culture. Before discussing the specific methods utilised in the experiments described in this thesis, basic principles regarding the culture environment will be introduced.

Substrate.

The majority of cells need to spread out on a substrate (solid, semi-solid or liquid) in order to proliferate (Fisher and Solursh, 1979). The solid substrates used are generally glass or plastic, although there is some experience with metallic surfaces such as stainless steel (Birnie and Simons, 1967). Cell proliferation will be inhibited if there is inadequate spreading owing to poor adhesion or overcrowding. In some cases attachment and cell growth can be improved by pretreatment of the substrate for example with collagen (Elsdale and Bard, 1972) or adding fibronectin to the culture medium (Gilchrest et al, 1980).

Incubation Conditions.

Atmospheric, or lower, oxygen tensions are preferable for most cell cultures (Cooper et al, 1958). The situation with regard to carbon dioxide is more complex as illustrated by the following equation:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

Bicarbonate (HCO_3^-) has a low dissociation constant with the cations available in the culture medium and it will therefore tend to reassociate, forming sodium bicarbonate, leaving the culture medium acid. The fall in pH that occurs as a result of increasing atmospheric CO_2 is neutralised by increasing the bicarbonate concentration of the culture medium. Each medium therefore has a recommended bicarbonate concentration and CO_2 tension to achieve the correct pH. Buffers such as HEPES can control pH within the physiological range (Good et al, 1966), although CO_2 remains essential, since the absence of it, or ultimately HCO_3^-, appears to restrict cell growth.

The optimal temperature for cell culture is dependent on the body temperature of the animal from which the cells were obtained, and incorporation of a safety factor to allow for minor errors in incubator regulation. Regulation of temperature should be kept within +/- 0.5 C, and temperature should be kept constant both in time and in different parts of the incubator. An even temperature distribution is achieved if the air is circulated with a fan, and cultures should be placed on perforated shelves, not on the floor of, or touching the sides of, the incubator.

Media and Supplements.

There have been major advances in culture media preparation, moving away from the "natural" media such as embryo extract and lymph, to the development of more complex "defined" media containing essential amino acids, vitamins and salts eg. Eagle's minimum essential medium (MEM) (Eagle, 1959); RPMI 1640 (Moore et al, 1967). Most cell lines grow well at a pH of 7.4 and therefore culture medium will need to be buffered if there is over production of CO_2 and lactic acid leading to a fall in pH, or if there is release of CO_2 (eg. open dishes) causing pH to rise. Bicarbonate is the buffer traditionally employed, in spite of its poor buffering capacity. HEPES is a

much stronger buffer in the pH range 7.2-7.6, and it is commonly used at 10 or 20 mM.

The essential amino acids plus cysteine and tyrosine are required by cultured cells, although the requirements will vary from one cell to another. Glutamine is required by most cells as a source of energy and carbon (Reitzer et al, 1979). Most cell cultures require supplementaion of the media with serum, and the one most commonly used is calf serum. Serum contains important proteins including albumin (Iscove and Melchers, 1978), globulins (Tozer and Pirt, 1964) and α_2-macroglobulin, a trypsin inhibitor (DeVonne and Mouray, 1978). Platelet-derived growth factor (PDGF) is one of the major growth factors in serum (Lechner et al, 1981), with factors such as epidermal growth factor (EGF) and insulin-like growth factors being present in smaller amounts (Gospodarowicz et al, 1978). Serum also contains hormones such as insulin, which promotes uptake of glucose and amino acids (Kelley et al, 1978), and cortisol, which promotes cell attachment and proliferation (Guner et al, 1977). There are, however, certain disadvantages associated with serum supplementation in that the actions of a number of minor constituents have not been characterised, and serum varies between batches. The effects of fetal calf serum on amnion and chorion cell cultures is discussed in detail in section 2.2.2.

2.2 Amnion and Chorion Dispersed Cell Method.

Described below are the techniques employed throughout this thesis.

2.2.1 Tissue Preparation.

Fetal membranes were collected directly in theatre from women undergoing elective caesarean section. Under sterile conditions the membranes were trimmed from the placenta, leaving a 1 cm margin, and transported in a sterile container (Sterilin) in Dulbecco's phosphate buffered saline solution (without calcium, magnesium or sodium bicarbonate) (Gibco, UK: 14190-094). The Dulbecco's phosphate buffered saline solution (DPBS) contained heparin (Leo Labs.) at a concentration of 10u/mL. The following procedures were carried out in a Microflow Biological Class 2 Safety Cabinet (MDH Ltd, Walworth Road, Andover, Hampshire, England). The amnion and chorion were separated and washed three times with DPBS. Any large blood clots were removed with fine tissue forceps. Fetal membranes that were meconium stained were not used and any "fibrous" tissue was discarded. The amnion and chorion were separated manually and any excess decidua was peeled from the chorion with fine tissue forceps. The tissues were weighed to enable calculation of the correct volume of digestion medium (see later). The tissues were soaked separately in 100 mL DPBS containing gentamicin (Sigma, UK G 1014) 40 mg/500 mL and amphotericin B (Sigma, UK A 9528) 2.5 mg/500 mL for one hour. The tissues were then washed four times with DPBS, using approximately 50 mL each time. The steeping and rinsing procedures were carried out to ensure that prostaglandins released secondary to the trauma of tissue collection were eliminated from the cultures. Using skin graft blades (BNO 7191/2A) the tissues were shredded into 3-4 mm pieces.

Chorion Pre-Incubation.

This was performed in order to remove as many red blood cells as possible. The chorion was transferred to a 250 mL Erlenmeyer flask (Corning Bibby 25600) containing a stirring magnet (Aldrich Co. Z16840/8). 100 mL of Digestion Medium was added. The flask was stirred at setting 1 on a magnetic stirrer for 10 minutes at 37 C. Taking care to retain all the tissue the digestion medium was poured off.

Tissue Digestion.

The amnion was transferred to a separate Erlenmeyer flask containing a stirring magnet. Digestion Medium was then added to both flasks: 5 mL per gram of amnion and chorion tissue (total approx. 100 mL). The flasks were stirred on the magnet stirrer (Bell Stir Multi-Stir 4; Bellco Biotechnology) at setting 1 for 40 minutes at 37 C.

There are a number of enzymes available for tissue digestion and disaggregation including trypsin, collagenase, elastase and DNAase. Trypsin achieves the most complete disaggregation but may damage the cells, whereas collagenase is less harmful to the cells at the expense of incomplete digestion (Freshney, 1987). Trypsin is the most commonly used enzyme and it is important to minimise exposure of the cells to preserve maximum viability. The action of trypsin is neutralised by the serum in complete culture medium, and where serum-free medium is employed, a trypsin inhibitor must be added. DNAase is used to disperse DNA from lysed cells since DNA will promote reaggregation and impair proteolysis. Both trypsin and DNAase were present in our digestion medium (section 2.2.2).

Cell Harvest.

Each tissue was filtered through a 0.16 mm nylon mesh and the filtrate, containing the cells, collected in a sterile container (Sterilin). Amnion and chorion were each divided in to three separate lots and the cells dispersed mechanically using an adapted Eppendorf pipette. The yield was harvested via the mesh into the sterile container and complete culture medium (approx. 30 mL) was added to the remaining tissue and the mechanical dispersal repeated three times. The total volume of harvested cells suspended in complete culture medium was transferred to 50 mL aliquot tubes (Falcon 2098) and centrifuged at 1500 rpm (MSE centrifuge) for 10 minutes. The supernatant was decanted and the pellets combined and resuspended in a smaller volume of complete culture medium (approx. 30 mL). As discussed above the culture medium will quench any further cell digestion. The process of centrifuging, decanting and resuspension was repeated a further two times and on the last occasion, after decanting the supernatant, the cells were resuspended in a fixed volume of complete culture medium (CCM) prior to counting.

Cell Count.

The amnion was suspended in 10 mL of CCM and the chorion in 20 mL of CCM. The difference in volume accounts for the fact that the amnion cells give a cleaner culture whereas the chorion cultures tend to have more debris in them. The cells were counted to assess number and viability using Trypan Blue exclusion. Using an Eppendorf pipette (Gilson) 50 µL of cells were mixed with 50 µL of Trypan Blue (Gurr Microscopic Materials, supplied via MERCK). An aliquot was transferred to a 0.1 mm Haemocytometer (British Standard 748, Weber Scientific International Ltd.), covered with a cover slip and counted under the microscope. Trypan is able to cross the membrane of dead cells which therefore stain blue. The absence of blue staining indicates viable cells. Having calculated the number of cells in a fixed aliquot the total

cell preparation was diluted down in complete culture medium to give approximately 4 x 10^6 cells per mL of CCM. 50 µL of this cell preparation will therefore give a cell density of 2 x 10^5 per well (final incubation volume 1 mL) in a standard 24 well plate (Corning Bibby, 25820).

2.2.2 Media Preparation.

The following solutions were all prepared under sterile conditions in the Microflow Biological Safety Cabinet.

Digestion Medium.

The digestion medium was prepared on the morning of each experiment (shelf-life: 6-8 hours), and is based on that described by other investigators (Jones et al, 1989; Riley et al, 1992). The ingredients are culture medium RPMI 16/40 (Gibco, UK: 52400/025); Trypsin (Sigma, UK: T 8253) and DNA-ase (Sigma, UK: DN-25). RPMI 16/40 contains L-Glutamine and 25mM HEPES Buffer and is stored at 5 C (shelf-life 6-9 months). The digestion medium was prepared in volumes of 200 mL DNAse, obtained in powder formulation, was dissolved in 10 mL of the RPMI and sterile filtered via a 0.22µm filter back in to the total volume of RPMI (200 mL) to give a final concentration of 0.02%. Trypsin, also obtained in powder formulation, was then added to the solution, final concentration 0.5%, and allowed to dissolve at 37 C which took approximately 1 hour.

Complete Culture Medium.

Complete culture medium was freshly prepared for each cell preparation and again it was prepared in volumes of 500 mL. The following agents were added to 440 mL of culture medium RPMI 16/40 (Gibco): 50 mL of bovine fetal calf serum (heat inactivated); 5 mL Penstrep solution, containing 5000IU Penicillin and 5000μg Streptomycin per mL; and 5 mL growth factors, containing insulin 25 mg, transferrin 25 mg and sodium selenite 25 μg. The bovine fetal calf serum (Gibco: 10108066) was obtained in 100 mL volumes and stored at -20 C. The Penstrep solution (Gibco: 04305070H) was also stored at -20 C. The growth factors (Sigma: I 1884), a lyophilised preparation, were reconstituted in 50 mL of sterile water (Elgastat purified system) and stored in 5 mL aliquots at 5 C. The formula for the above preparation is again based on that used by other workers (Jones et al, 1989; Riley et al, 1992) with some minor modifications (Gibb and Lavoie, 1990).

The culture medium used in our experiments contains 10% fetal calf serum. A number of experiments have been performed investigating the influence of fetal calf serum (FCS) on the responsiveness of cell cultures. It has been demonstrated that basal PGE_2 production by amnion and chorion is reduced by 75% and 35% respectively when cultures are performed in the absence of FCS (Edwin and Mitchell, 1992). The difference in basal PGE_2 production between cultures with or without FCS could be fully or partially overcome by the addition of arachidonic acid in amnion and decidual cells, but not in chorion cells. This suggests that arachidonic acid is not the only factor in FCS regulating cell responsiveness. In the same experiments the responsiveness of cells to stimulatory agents such as epidermal growth factor, ionomycin and interleukin-1β was influenced by the composition of the culture medium.

The effect of different concentrations of FCS supplementation has also been investigated by Gibb et al (1986). They found that the morphological appearance of

chorion laeve cells was quite different in cultures using 0.1% FCS compared with 10% FCS. In cultures using 0.1% FCS the cells were clumped, and remained so throughout the duration of the culture period. However, in the presence of 10% FCS, there was no cell clumping and a single, evenly dispersed layer of cells was achieved which reached confluence at day five. In these experiments the protein and DNA content of the cultures was increased in the presence of 10% FCS, in contrast to 0.1% FCS which did not stimulate either. Also, the increase in thymidine incorporation into the cells was greater in the presence of 10% FCS, and all of these findings taken together confirm cell replication in the cultures supplemented with 10% FCS.

These findings highlight the influence that culture medium constituents can have on the morphological and biochemical properties of cell cultures, and the need to take this into account when interpreting *in vitro* data.

Steeping Solution.

Dulbecco's phosphate buffered saline (DPBS) was stored at room temperature. Gentamicin and amphotericin B were added to 500 mL of DPBS. Gentamicin (Sigma: G 1014) was obtained in sterile solution, 50 mg/mL, and stored at 5 C. Using a 1 mL Eppendorf pipette, 800 µL of gentamicin solution was added to the DPBS giving a final concentration of 40 mg/500mL. Amphotericin B (Sigma: A 9528) was obtained in powder formulation in 100 mg lots and made up in sterile water to a concentration of 10 mg/mL. This was stored at 5 C and, using a 0.5 mL Eppendorf pipette, 250 µL added to the DPBS giving a final concentration of 2.5 mg/500mL. The steeping solution was stored at 5 C and has a shelf life of 3 weeks.

2.3 Tissue Explant Culture.

Chapter three describes experiments investigating changes in prostaglandin synthesis and metabolism by amnion and chorion respectively in association with labour. Explant cultures were performed so that relatively intact tissue could be studied. The culture procedures for these experiments are outlined below.

Culture preparation.

Fetal membranes were collected at the time of elective caesarean section or immediately following spontaneous labour and delivery in the same manner as described in section 2.2.1 (paragraph 1). The initial procedures in this experiment were exactly the same as for the cell culture experiments up to and including the steeping in antibiotics and the thorough washing in DPBS thereafter.

Amnion Tissue Culture.

Standard 24 well plates (max. volume 2 mL: Corning-Bibby 25820) were also used for these experiments. 900 μL of complete culture medium was pipetted (Eppendorf pipette) in to each of 8 wells in the standard plate. This was then placed in the incubator (CO_2 gased and humidified; Scotlab-VSL) whilst the amnion discs were prepared. The amnion was placed on a Petri dish (150 mm diameter; NUNC intermed) and, using a size 12 mm cork borer, eight discs were cut out. Each disc was suspended on a 1 cm^2 piece of sterile capillary matting and then submerged in a well of the standard plate.

Chorion Tissue Culture.

Four standard plates per subject were used for the chorion experiments. 800 μL of complete culture medium was pipetted in to each of 8 wells per plate. These plates were put in the incubator while the chorion discs were prepared. The chorion was placed on a Petri dish as above and 32 discs of 9 mm diameter were cut with a cork borer. The discs were suspended on sterile capillary matting and submerged in the culture medium in the wells.

Preparation of capillary matting.

Fybamat capillary matting (B&Q, Edinburgh UK) was cut in to 1 cm squares. 150-200 squares were placed in a 250 mL sterile container (Sterilin). These were steeped in 2% Shield (Jeyes Hygiene) for approximately 1 hour and then rinsed thouroughly under hot water in a sieve until there was no foaming. The squares were steeped in 70% ethanol overnight. The ethanol was aspirated until the squares were almost dry and they were then steeped in DPBS for approximately 15 minutes. This was aspirated until the squares were dry and the containers were left in the safety cabinet overnight with the tops off to dry completely.

2.4 Culture Treatments.

2.4.1 Cell culture treatments.

Actinomycin D (Sigma, UK: A 1410), stored at 5 C.

Cycloheximide (Sigma, UK: C 6255), stored at 5 C.

Genistein (Sigma, UK: G 6649), stored at -20 C.

Staurosporine (Sigma, UK: S 4400), stored at 5 C.

The above agents were all obtained in powder formulation. They were each made up in complete culture medium to 10 times the concentration required in the experiments in the sterile safety cabinet and then stored at 5 C overnight. Volumes of 100 μL were then added to the wells which gave the correct final concentration per mL.

2.4.2 Tissue explant treatments.

Arachidonic Acid (Sigma, UK: A9548)

Indomethacin (Sigma, UK: I7378)

Phorbol Myristoyl Acetate (Sigma, UK)

Dexamethasone (Sigma, UK: D1756)

Progesterone (Sigma, UK)

RU 486 (Roussel Laboratories, UK)

The above agents were all obtained in powder formulation and made up in concentrated ethanol solution, with the exception of arachidonic acid, for storage at -20 C. Arachidonic acid is insoluble in ethanol and was therefore made up in complete culture medium and stored at -20 C. Working solutions were obtained by diluting the concentrated solutions in complete culture medium, again to ten times the concentration required for the experiments. These working solutions were stored at -20 C and thawed prior to addition to the cell culture systems. Again, a volume of 100 µL was employed to achieve the correct final concentration per well. All solutions were frozen and thawed once only; any excess was discarded.

PGE_2 (Upjohn Ltd. Lot 23007).

PGE_2 was obtained as a sterile solution at a concentration of 10 mg/mL in ethanol. In the safety cabinet under sterile conditions 10 µL of this solution was diluted in 20 mL of complete culture medium to give a concentration of 5 µg PGE_2 per mL. This was stored in 1.4 mL aliquots at -20 C and was thawed immediately prior to use (these aliquots were frozen and thawed once only). Addition of 100 µL to each well gave a final concentration of 500 ng PGE_2 per well. The maximum amount of ethanol tolerated per well is 0.5 µL (ie. 0.05%). The above dilution results in 0.05 µL of ethanol per 100 µL of PGE_2 solution which equates to 0.005% per well (1 mL volume). The remaining original solution was stored in a sterile glass vial at -20 C.

$PGF_{2\alpha}$ (Upjohn Ltd. Lot 22322).

$PGF_{2\alpha}$was obtained as a sterile solution at a concentration of 5 mg/mL in aqueous solution. In the safety cabinet under sterile conditions 20 µL of this solution was diluted in 20 mL of complete culture medium to give a concentration of 5 µg $PGF_{2\alpha}$ per mL. Addition of 100 µL of this to each well gave a final concentration of 500 ng

$PGF_{2\alpha}$ per well. This was stored in 1.4 mL aliquots at -20 C and thawed immediately prior to use (these aliquots were frozen and thawed once only). The remaining original solution was stored in a sterile glass vial at 5 C.

2.5 Oximation and Storage of Samples.

Upon completion of all cell and tissue explant experiments 0.5 mL of culture medium was aspirated from each well into a Sarstedt microtube (2 mL capacity: 72694007) and oximated with an equal volume of methyl oximating solution to promote formation of stable methyl oxime compounds (see radioimmunassay details). These samples were thoroughly mixed, stored at room temperature for 24 hours and then at 5 C pending radioimmunoassay.

Methyloximating Solution (Mox B).

This was prepared in 2 L volumes. 164 g of Sodium acetate (Sigma, UK; S8750) and 20 g of methoxyamine hydrochloride (Sigma, UK; M1139) were weighed out and approximately 1200 mL of distilled water and 200 mL of ethanol added. The final concentrations of these reagents were 1 M and 0.12 M respectively. These were mixed until dissolved in the fume cabinet (Morgan & Crundy). The pH was measured (Orion: 520) and adjusted to 5.6-5.8 with hydrochloric acid (5M) and the total volume made up to 2 L with distilled water. This solution was stored in a glass bottle at room temperature in the fume cabinet.

2.6 Radioimmunoassay.

2.6.1 Introduction.

A competitive-binding radioimmunoassay was employed. This works along the following principles. The prostaglandin (antigen) of unknown quantity present in the sample is mixed with ^{125} I-labelled prostaglandin of known quantity. The first antibody is then added to this solution and the labelled and unlabelled prostaglandins compete with each other for binding to the antibody. If the level of unknown prostaglandin in the sample is high it will bind preferentially with antibody and the amount of radio-labelled antigen-antibody complex will be correspondingly low. Therefore, a low count of radioactivity represents a high level of unknown prostaglandin in the original sample and vice versa. A second antibody, raised against the first antibody and attached to magnetic particles, is then added to the solution and this binds to antigen-antibody complexes, both labelled and unlabelled. The solution is then attached to a magnet to separate the free and bound antigen. The supernatant, containing labelled and unlabelled free antigen, is poured off. The antigen-antibody complexes are counted by a gamma counter.

A standard curve is constructed to enable estimation of the quantities of unknown antigen present in the samples being measured. This is done by treating a range of samples of antigen of known concentration with radiolabel and antibody in exactly the same manner as described above.

All samples obtained from the explant and cell culture experiments were oximated with methyloxime solution prior to storage pending radioimmunoassay. The resultant

conversion of the prostaglandins to their corresponding methyl oximes prevents sample decomposition during storage or in the course of the assay since these methyl oxime derivatives are much more stable. The process of methyloximation described earlier (page 79) gives greater than 95% conversion of prostaglandins to their methyl oximes when treated overnight at room temperature. The reason for obtaining greater stability of these compounds is that there have been problems with prostaglandin analysis in the past owing to the inherent instability of the β ketol ring structure which is present in prostaglandins of the E series. This has resulted in the detection of erroneously high levels of prostaglandins of the A series. Conversion to methyl oximes does not interfere with assay of those prostaglandins such as $PGF_{2\alpha}$ which cannot form methyl oximes. The antiserum is therefore raised against the methyl oxime form.

In summary, up to 50 μL of oximated culture medium was assayed. The labels employed are the iodinated (^{125} I) methyl oximes of PGE_2, $PGF_{2\alpha}$, PGEM and PGFM. The prostaglandin methyl oximes are coupled to a tripeptide Pro-gly-tyr through the nitrogen in the proline ring. This linkage is distinct from the amide linkage present in the original antigen against which the antiserum is raised. This ensures that the antiserum does not have a higher affinity for the label than it has for the compound being measured and results in a high sensitivity assay. Antisera to the respective antigens were raised in a rabbit. A second antibody, raised in a donkey, was used to separate antigen-antibody complexes from free antigen in a magnetic separation procedure.

2.6.2 Assay Procedure.

All samples were assayed in duplicate. Sarstedt polypropylene tubes (55532) were numbered and set up on racks supplied by Amersham Radiochemical Company.

1. Preparation of the standard curve.

The standards were supplied by Upjohn and stored at concentrations of 20 µg/mL in ethanol at -20 C. Stock dilutions were made up in 20% Mox buffer to maintain stability and stored at 5 C. These dilutions have a shelf-life of nine months. Working dilutions were prepared by making a 1:10 dilution of the stock dilutions in 20% Mox buffer to give the following range of concentrations for use in the assay: 10, 20, 40, 80 160, 312, 652, 1250, 2500 and 5000 pg/mL. The working dilutions were also stored at 5 C and were renewed every four months.

2. In general 50 µL of each sample to be assayed was used. Where the concentration of prostaglandins or their metabolites was expected to be high (eg. PGEM and PGFM in the chorion experiments) a 25 µL sample was assayed. In order to maintain a stable pH, and so optimise antibody binding, the concentration of Mox in the samples was kept constant at approximately 20-25%. The samples already contained 50% Mox B (NB storage procedure). Therefore, a 50 µL sample would be made up to 100 µL with 50 µL of assay buffer; and a 25 µL sample would be made up to a volume of 100 µL with 25 µL of assay buffer and 50 µL of 20% Mox buffer.

3. 100 µL of antiserum dilution was added to all tubes except the non-specific binding (NSB) and total count (TC) tubes. Non-specific binding was always <2% for all prostaglandin assays.

4. 100 µL of radiolabel (tracer) was added to all tubes to yield approx 15,000 counts per minute/100 µL.

5. The racks were clipped on to a shaker (Amersham N4201) for one cycle at full speed.

6. The racks were covered and allowed to incubate overnight at room temperature.

7. The total count tubes were set aside. 250 µL of second antibody (attached to magnetic particles) was added to all the other tubes.

8. The tubes were mixed on the shaker for one cycle at full speed and incubated for 30 minutes at room temperature.

9. The racks were clipped to a magnet for 15 minutes to allow separation of the bound and free antigen to occur.

10. With the racks and magnet clipped together the supernatant was decanted and the racks allowed to drain on to paper towels for a few minutes.

11. The racks were removed from the magnets and 250 µL of assay buffer containing 1.25% Triton X/80 ml assay buffer was added to each tube. The samples were mixed on the shaker for one cycle at full speed and free and bound antigen reseparated with the magnet for 15 minutes at room temperature.

12. The supernatant was decanted as before and the tubes drained for a further 15 minutes.

13. The assay tubes were counted for two minutes to assess the amount of bound ligand using the appropriate programme on the Packard Gamma Counter.

2.6.3 Cross Reactivities.

The cross reactivities to PGE_2 and $PGF_{2\alpha}$ antisera are displayed in table 2.1, and those for PGEM and PGFM antisera (raised against the 13,14-dihydro-15 keto PGE_2 derivative, and the 13,14-dihydro-15 keto $PGF_{2\alpha}$ derivative respectively) are displayed in table 2.2. Cross reactivity was determined by the amount of prostaglandin that caused 50% inhibition of binding of the appropriate labelled prostaglandin to the antibody (Kelly et al, 1986).

The sensitivity of the assay (the amount distinguishable from zero with a 95% confidence limit) was 2 pg in all assays. This gives a limit of detection of approximately 40 pg per well. The intra- and inter-assay coefficients of variation are illustrated in table 2.3 (Kelly et al, 1986; Kelly et al, 1992).

Antiserum	Compound	Cross Reactivity
PGE_2	PGE_1	53%
	PGE_3	31%
	PGB_2	0.2%
	20-methyl PGE_2	31%
	19-hydroxy PGE_1	17.8%
	19-hydroxy PGE_2	3.7%
	20-hydroxy PGE_2	2.9%
	8-iso PGE_2	0.25%
	15-keto PGE_2	0.25%
	All other prostaglandins	<0.2%
$PGF_{2\alpha}$	$PGF_{1\alpha}$	7.2%
	$PGF_{3\alpha}$	2.9%
	$PGF_{2\beta}$	3.5%
	PGE_2	1.1%
	6-oxo $PGF_{1\alpha}$	1.05%
	13,14-dihydro $PGF_{2\alpha}$	1.0%
	All other prostaglandins	<0.2%

Table 2.1. Cross reactivities to PGE_2 and $PGF_{2\alpha}$ antisera.

Antiserum	Compound	Cross Reactivity
13,14-dihydro-15-keto PGE_2	15-keto PGE_2	11.7%
	15-keto PGE_2	0.94%
	13,14-dihydro $PGF_{2\alpha}$	0.19%
	13,14-dihydro $PGF_{2\alpha}$	0.11%
	All other prostaglandins	<0.02%
13,14-dihydro-15-keto $PGF_{2\alpha}$	$PGF_{2\alpha}$	4.0%
	13,14-dihydro $PGF_{2\alpha}$	2.0%
	6,15-dioxo-13,14-dihydro $PGF_{1\alpha}$	0.35%
	13,14-dihydro-15-keto PGE_2	0.12%
	PGD_2	0.08%
	$PGF_{1\alpha}$	0.07%
	PGE_2	0.04%
	All other prostatglandins	<0.01%

Table 2.2. Cross reactivities to 13,14-dihydro-15-keto PGE_2 and 13, 14-dihydro-15-keto $PGF_{2\alpha}$ antisera.

	PGE_2	$PGF_{2\alpha}$	13,14-dihydro-15-keto PGE_2	13,14-dihydro-15-keto $PGF_{2\alpha}$
Intra-assay Precision	12.3%	10.5%	8.4%	7.3%
Inter-assay Precision	13.3%	13.9%	13.9%	13.3%

Table 2.3. Intra- and inter-assay coefficients of variation.

2.6.4 Preparation of buffers.

Tris 1 M Buffer. (Tris(hydroxymethyl) methylamine)

Made up in 1 litre volumes. 121.1 g of Tris (BDH) is dissolved in approximately 800 mL of distilled water. The pH is adjusted to 7.3 with concentrated hydrochloric acid (BDH) (approx. 60 mL required). This is then made up to the final volume of 1 L with distilled water and stored at 5 C. Tris acts as a secondary buffering system and helps to maintain a constant pH and so maximise antigen-antibody binding.

Assay Buffer [Phosphate Gelatin Buffer (BDH British Drug Houses) + Tris].

Made up in 4 litre volumes. The ingredients are NaCl, 36 g; Na_2HPO_4, 34.4 g; Na_2HPO_4 $2H_2O$, 24.32 g; NaN_3, 2g; Gelatin, 4g; Tris buffer 1M (pH 7.3), 400 mL. The gelatin is dissolved in 500 mL of distilled water by heating. This is removed from the heat and 2.5 L of distilled water plus the dry salts are added and stirred until dissolved. 400 mL of the Tris buffer is added (final concentration 100mM) and the pH adjusted to 7.8 with 10M NaOH. This is made up to the final volume with distilled water and stored at 5 C.

Triton-X-100 1.25%.

1.25 mL of Triton-X-100 (BDH) is dissolved in 100 mL of assay buffer at room temperature and stored at 5 C.

20% Mox Buffer = 20 mL Mox B
0.4 mL 1.25% Triton X
79.6 mL Assay Buffer

10% Mox Buffer = 10 mL Mox B
4 mL 1.25% Triton X
86 mL Assay Buffer

2.6.5 Preparation of tracer and antibodies.

Tracer.

The tracers were prepared and stored in ethanol at 5 C. They were then diluted in 10% Mox buffer to yield approximately 15,000 counts per 100 µL.

Antibodies.

The first antibody was raised in a rabbit and this was stored in lyophilised form at -20 C. The antibody was reconstituted in assay buffer (concentration 1:100) and stored at 5 C. Fresh working dilutions, ranging from 1: 15, 000 - 40, 000 were made up in assay buffer for each assay.

The second antibody, donkey anti-rabbit anti-serum, was linked to magnetic particles. Neat solution was stored at 5 C and working dilutions were made up in assay buffer containing 1 mL 1.25% Triton-X per 80 mL assay buffer to give a dilution of 1:16. Triton-X prevents the particles from sticking to the assay tubes.

2. 7 Amniotic Fluid Collection and Preparation.

Amniotic fluid was collected at elective caesarean section by transmembranous amniocentesis and immediately with delivery of the baby following spontaneous labour under aseptic conditions. The fluid was centrifuged at 2000 rpm for 20 minutes at room temperature to remove any excess debris and divided in to aliquots for storage at -20 C prior to use in the cell culture experiments. The fluid was thawed on the morning of each experiment and centrifuged once more as described above and then filtered through 0.22 μm mesh to remove any contaminating particulate matter prior to addition to the cell culture system.

There is no method of collection of amniotic fluid from women in spontaneous labour, other than transabdominal amniocentesis, that will ensure a sterile sample. As this is not ethically or practically realistic in our centre we collected amniotic fluid at the time of delivery under aseptic conditions. In so doing, only women who had laboured spontaneously without oxytocin augmentation were sampled. The amniotic fluid had therefore been in contact with the maternal genital tract at the time of delivery raising the concern of bacterial contamination. It has been demonstrated that potentially pathogenic bacteria can increase arachidonic acid metabolism by amnion cells. However, Lactobacillus, which forms the normal vaginal flora does not (Bennett et al,

1987). There is also the potential that bacteria themselves may synthesise prostaglandins but it has been established that although they are capable of incorporating arachidonic acid, common genital tract pathogens are unable to metabolise arachidonic acid via cyclooxygenase, lipoxygenase, or epoxygenase pathways (Gulbis et al, 1981; Bennett et al, 1992a).

It has been shown that bacterial products have a bi-phasic effect in prostaglandin production by amnion and decidua (Mitchell et al, 1991a), with low doses stimulating and high doses inhibiting prostaglandin synthesis. The results of the experiments in chapters four and five demonstrate that amniotic fluid had a consistent effect on prostaglandin production which was stimulated in a dose-dependent manner. In addition, the amnion and chorion cells in all experiments were maintained in culture for seven days, at the end of which time there was no evidence of bacterial infection suggesting that any increase in prostaglandin production by these cells is not the result of contaminating bacteria.

Background concentrations of PGE_2, $PGF_{2\alpha}$ and their respective metabolites PGEM and PGFM were established in all amniotic fluid samples and appropriate subtractions were made in the experiments described in chapters four and five. Estimation of background cytokine concentrations in the amniotic fluid was not performed as it would not be possible to make any distinction between cytokines which might be a cause of and those which might be the result of the spontaneous labour process.

Chapter Three

Verification of Methodology

3.1 Cell Density.

Methods.

Fetal membranes were collected at elective caesarean section (n=3) and amnion and chorion cell cultures prepared as described in section 2.1.1. Cells were plated out in standard 24 well plates at the following densities: 0.5, 1, 2, 4 and 8 $x10^5$/ mL. The plates were incubated in humidified 95% air; 5% CO_2 at 37 C, and all experiments were performed in duplicate. After 24 hours and seven days in culture, 0.5 mL of culture medium was aspirated from each well and oximated as described in section 2.4. The primary prostaglandins PGE_2 and $PGF_{2\alpha}$ and their respective metabolites PGEM and PGFM were measured by radioimmunoassay (section 2.6). All cultures had reached confluence by seven days.

Statistical Analysis.

Statistical analysis was performed using ANOVA. Data that were not normally distributed were log transformed prior to analysis.

Results.

In the 24 hour cultures PGE_2 was the major product of the amnion, and significantly more PGE_2 was produced at densities of 2, 4 and 8 $x10^5$ cells/ mL compared with 0.5 $x10^5$ cells/ mL (Fig. 3.1; $p<0.05$). There was no significant difference between the 24 hour and 7 day cultures in the concentration of PGE_2 produced at any cell density. $PGF_{2\alpha}$ was produced in similar concentrations at all cell densities (Fig. 3.1). Its production was significantly lower than that of PGE_2 at cell densities of 2, 4 and 8 $x10^5$ cells/ mL ($p<0.05$). Again, there was no significant difference between the 24 hour and 7 day cultures in the concentration of $PGF_{2\alpha}$ produced at any cell density.

There was no significant difference in PGEM production by chorion at any cell density between the 24 hour and seven day cultures. Significantly more PGEM was produced at a density of 8 $x10^5$ cells/ mL compared with 0.5 $x10^5$ cells/ mL at both incubation times (Fig. 3.2; $p<0.05$). Similarly, there was no difference in PGFM production at any cell density between the two incubation times. PGFM production by cell densities of 1, 2, 4 and 8 $x10^5$ cells/ mL was significantly greater than 0.5 $x10^5$ cells/ mL in the seven day cultures (Fig. 3.2; $p<0.05$). In addition, by the seventh day of culture PGFM production was significantly lower than PGEM production at all cell densities ($p<0.05$).

Production of primary prostaglandins and their metabolites at a density of 2 $x10^5$ cells/ mL was not significantly different from the higher cell densities, except in the case of PGE_2. However PGE_2 production had increased significantly at 2 $x10^5$ cells/ mL compared with 0.5 $x10^5$ cells/ mL. Cell viability data for the three cultures is illustrated in table 3.1.

Discussion.

All cell culture experiments described in the chapters four and five employ a cell density of approximately 2 $x10^5$ cells/ mL. This was chosen as it is practical allowing stimulation and inhibition of production to be studied with ease, avoiding grossly excessive concentrations of prostaglandins in the medium as may occur at higher cell densities. This is also in keeping with experiments performed by Jones et al, (1989) and Riley et al (1992). It is also within the range of densities (1 x 10^5 cells/ mL to 3 x 10^5 cells/ well) used by a number of other investigators (Olson et al, 1983b; Gibb et al, 1986; Edwin and Mitchell, 1992).

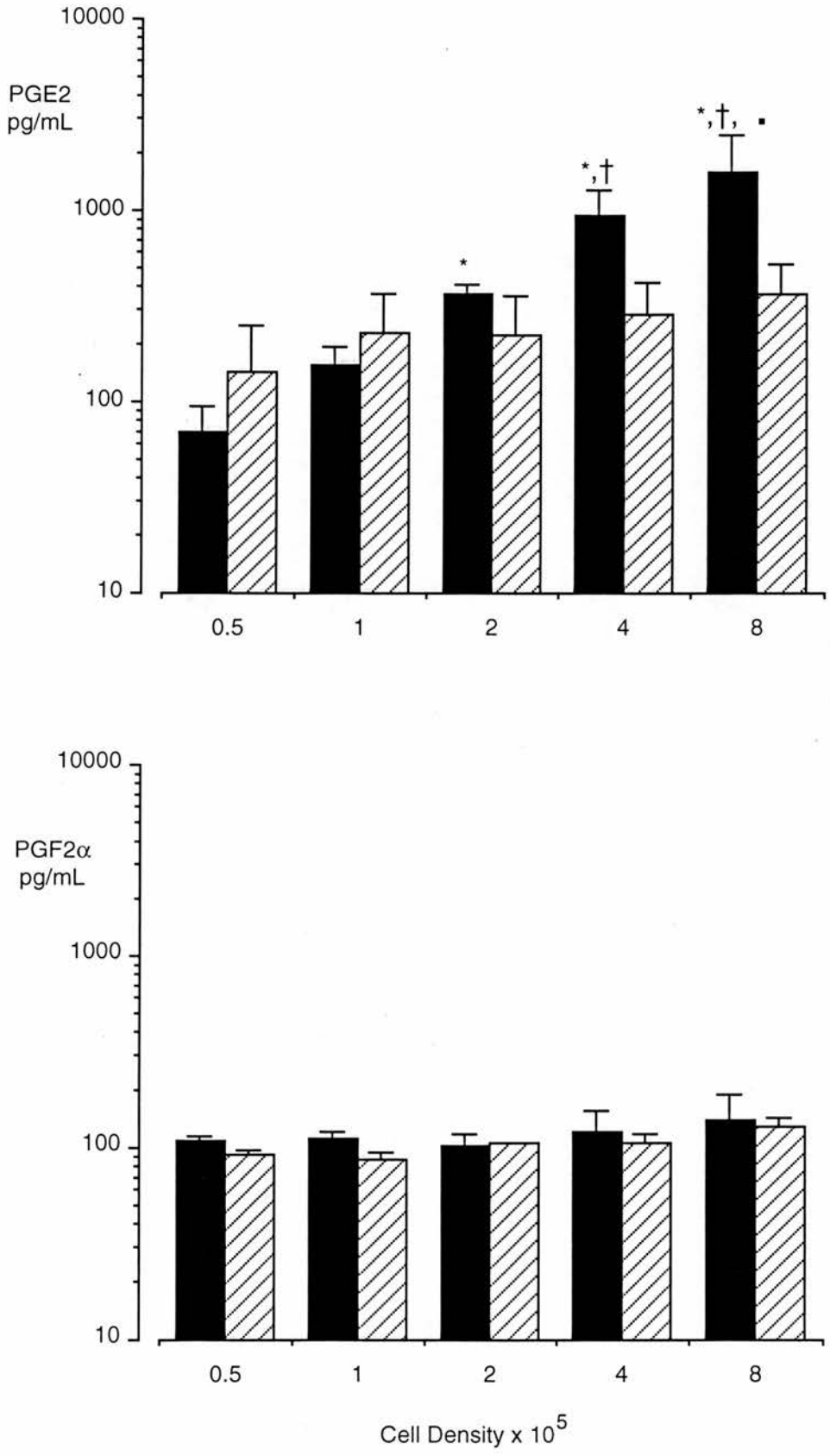

10000
PGE2
pg/mL
1000
100
10
*
*,†
*,†, ▪
0.5
1
2
4
8
10000
PGF2α
pg/mL
1000
100
10
0.5
1
2
4
8
Cell Density x 10^5

Figure 3.1

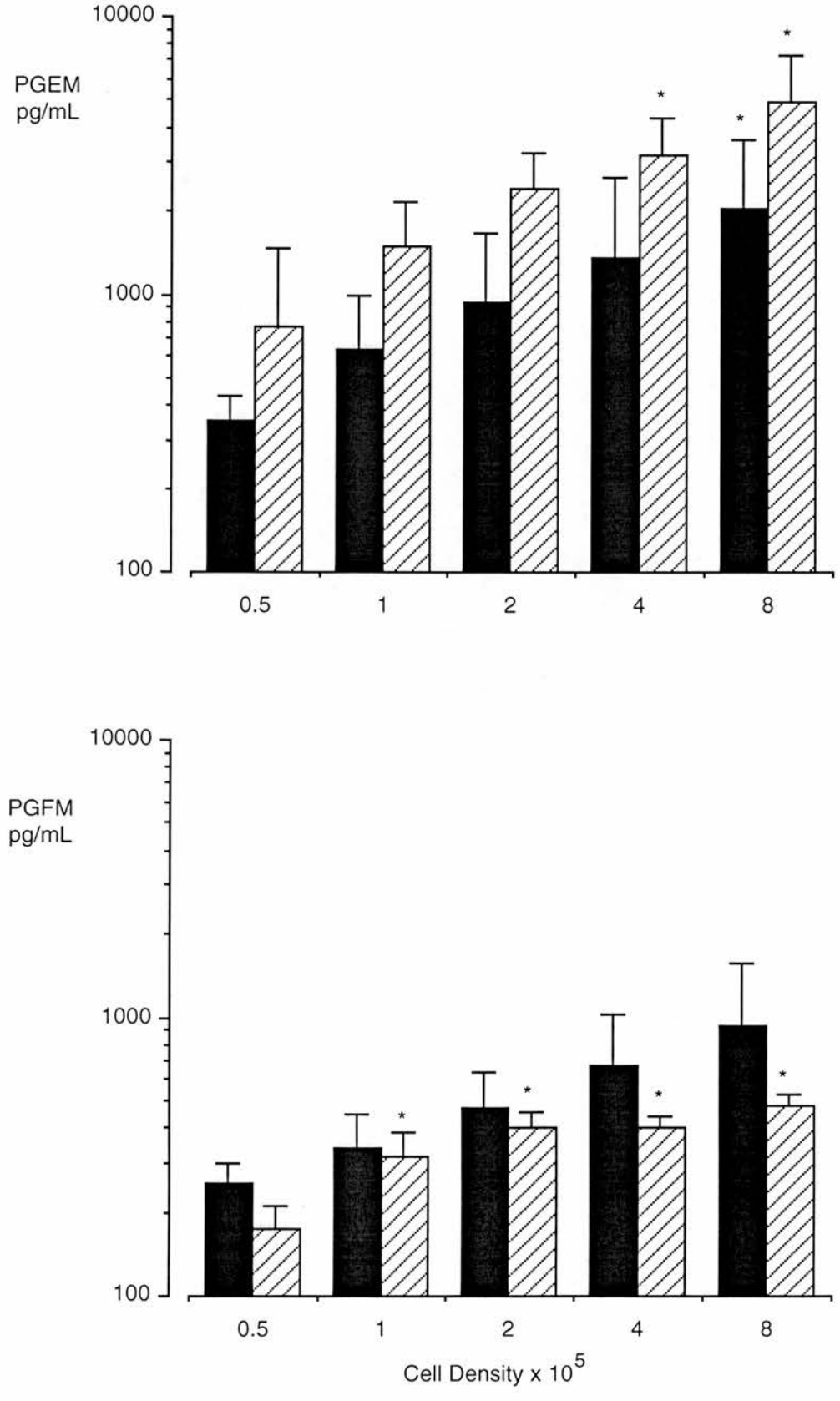

10000
PGEM
pg/mL
1000
100
0.5
1
2
4
8
*
10000
PGFM
pg/mL
1000
100
0.5
1
2
4
8
Cell Density x 10^5

Figure 3.2

	Culture				
	I	II	III	Mean	Coeff. Variation
Amnion Cells (24 hours)	91.3%	84.5%	86.5%	87.4%	4.0
Amnion Cells (7 days)	75.5%	74.3%	71.2%	73.7%	3.0
Chorion Cells (24 hours)	90.7%	89.6%	82.0%	87.4%	5.4
Chorion Cells (7 days)	86.3%	88.9%	82.3%	85.8%	3.9

Table 3.1. Cell viability data.

3.2 Duration of Culture.

It has been established by a number of studies that basal prostaglandin production, and the response to various stimulants can be influenced by the length of time the cells have been in culture before the experiments are performed. For example, it has been shown that basal PGE_2 production by amnion cells decreases during cell culture such that it may be undetectable by day seven (Gibb and Lavoie, 1990). In these experiments, the cells reached confluence between days five and eight of culture. Similarly, the response of amnion cells to various agents was shown to change with duration of culture. In these experiments EGF had no effect on freshly isolated cells, but stimulated PGE_2 production in confluent cells. Likewise the effect of dexamethasone on PGE_2 production switched from inhibition in fresh cells to stimulation in confluent cells. This stimulatory effect of dexamethasone on confluent cell PGE_2 production has been confirmed in other studies (Mitchell et al, 1988a; Potestio et al, 1988), and it has been suggested that this effect is most likely regulated at the level of COX (Potestio et al, 1988). The effects of dexamethasone on prostaglandin production are discussed in more detail in chapter three.

Further evidence supporting changes in cell responsiveness with culture duration is provided by the finding that in the first and second 24 hours of culture amnion and chorion cells are unresponsive to interleukin 1β, but by day five of culture PGE_2 production by both cell types was stimulated by this cytokine (Alnaif et al, 1994). A number of suggestions have been proposed by these authors to account for the differences. It may be that COX activity is reduced after the first 48 hours of culture, and it is only once this has occurred that the stimulatory effect of IL-1β can be seen. Alternatively receptor damage may occur during cell dispersal, but as receptor turnover

is achieved between 2 and 12 hours a response to IL-1β should be seen in the second 24 hours of culture if this were the explanation.

Lundin-Schiller et al (1991b) described prostaglandin production by chorion cells and the response to inflammatory mediators. Their cultures reached confluence on day three, and basal prostaglandin production was significantly greater at this time compared with day ten. This is in keeping with the results of Gibb et al (1988) who found that PGE_2 production by chorion cells was maximal on day one or two of culture, and then declined to low levels. Lundin-Schiller et al (1991) observed a large variation in basal prostaglandin production between tissues from different pregnancies, and this is a consistent feature of such cell culture work (Gibb et al, 1988; Alnaif et al, 1994). The responsiveness of the chorion cells to TNFα , IL-1β and endotoxin was significantly greater at day ten compared with day three of culture, reflected by increased PGE_2 production. Again these authors speculate that this may be due to recovery of receptors as the cultures progress. Alternatively, they suggest that there may be a relative increase in a subpopulation of chorion cells that are responsive to cytokines compared with other cells, after several days in culture.

The above findings illustrate the quantitative and qualitative changes that occur in cell culture systems with time, and the caution with which *in vitro* results must be interpreted in relation to the *in vivo* situation.

In view of the above we compared production of PGE_2, $PGF_{2\alpha}$ and their respective metabolites PGEM and PGFM by cells cultured for 24 hours and seven days and looked at the effect of the protein kinase C stimulator PMA, and the cytokine TNF_α on their production.

Methods.

Fetal membranes were collected at elective caesarean section (n=3) and amnion and chorion cell cultures prepared as described in section 2.2.1. They were plated out at densities of 0.5, 1, 2, 4 and 8 $x10^5$ cells/ mL. After an incubation period of 24 hours or seven days, in humidified 95% air; 5% CO_2 at 37 C, the following additions were made: 100nM PMA (to all cell densities), 1000u TNFα (to $2x10^5$ cells/ mL) or culture medium (to act as control). The cells were incubated for a further 24 hours, and then 0.5 mL of culture medium aspirated for oximation and radioimmunoassay as described in sections 2.5 and 2.6. All experiments were performed in duplicate.

Statistical Analysis.

Statistical analysis was performed using ANOVA. Data that were not normally distributed were log transformed prior to analysis.

Results.

PGE_2 and $PGF_{2\alpha}$ production by amnion cells cultured for 24 hours was significantly stimulated by PMA (Fig. 3.3, 3.4; $p<0.05$). Following seven days in culture PGE_2 production was only stimulated by PMA at a cell density of 8 $x10^5$/ mL, and the phorbol ester had no effect on $PGF_{2\alpha}$ production after this length of culture (Fig. 3.3, 3.4). PMA significantly stimulated PGEM production by chorion cells after 24 hour culture at all densities (Fig. 3.5; $p<0.05$), and PGFM production was stimulated at densities of 2, 4 and 8 $x10^5$ cells/ mL (Fig. 3.6; $p<0.05$). Following seven days in culture PMA continued to stimulate production of both metabolites at certain cell densities (Fig. 3.5, 3.6).

TNFα significantly stimulated PGE_2 production by amnion after 24 hours in culture (Fig. 3.7; $p<0.05$) and this stimulation was maintained after seven days in culture. $PGF_{2\alpha}$ production was not affected by TNFα at either time point (Fig. 3.7). However, baseline production of $PGF_{2\alpha}$ is very low compared with that of PGE_2. TNFα had not effect on PGEM production by chorion after 24 hours in culture, but in the seven day cultures PGEM production was stimulated, although this failed to reach significance ($p=0.057$). There was no effect on PGFM production at either time point (Fig. 3.7).

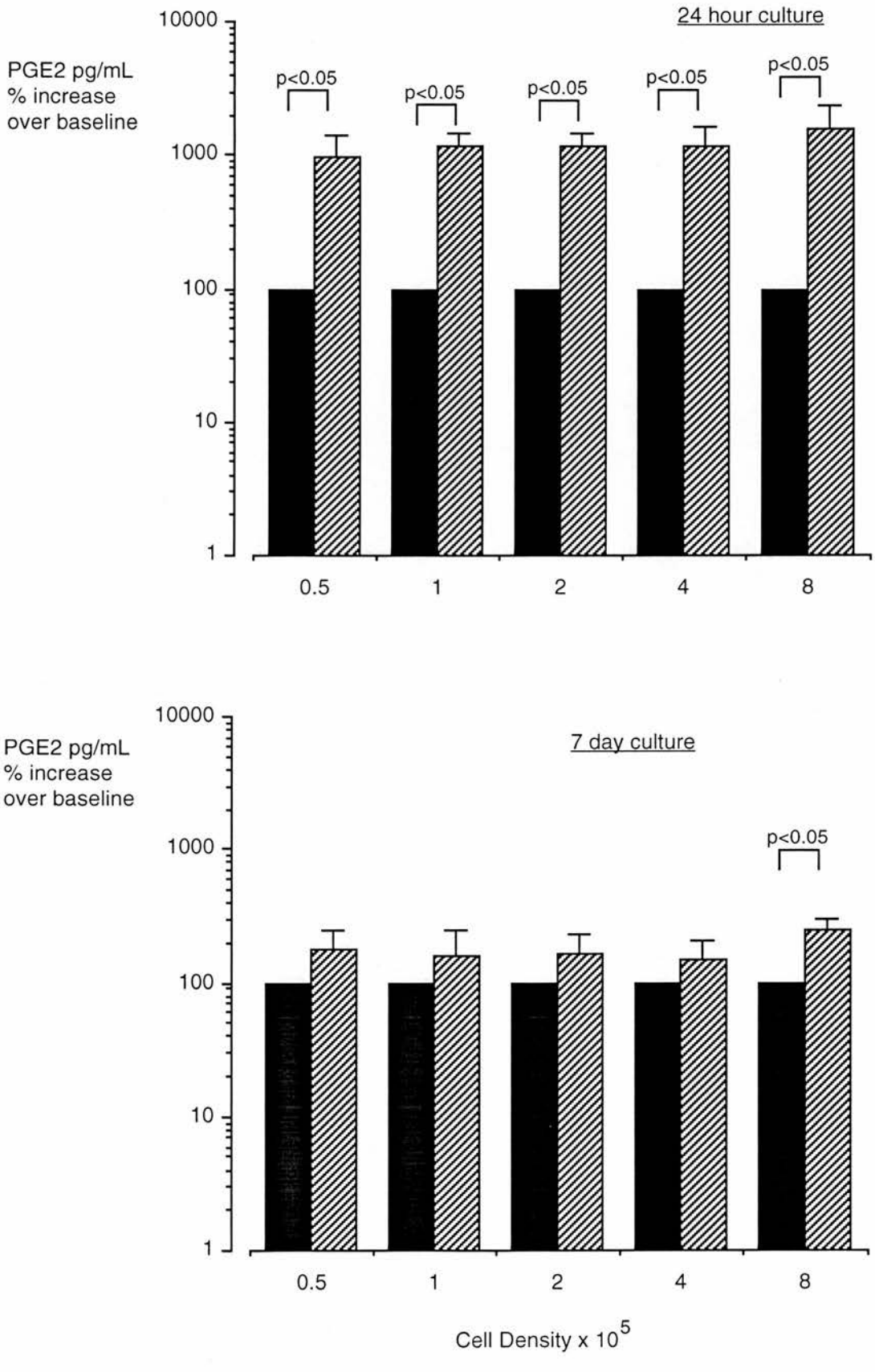

24 hour culture
PGE2 pg/mL
% increase
over baseline
10000
1000
100
10
1
p<0.05
p<0.05
p<0.05
p<0.05
p<0.05
0.5
1
2
4
8
7 day culture
PGE2 pg/mL
% increase
over baseline
10000
1000
100
10
1
p<0.05
0.5
1
2
4
8
Cell Density x 10^5

Figure 3.3

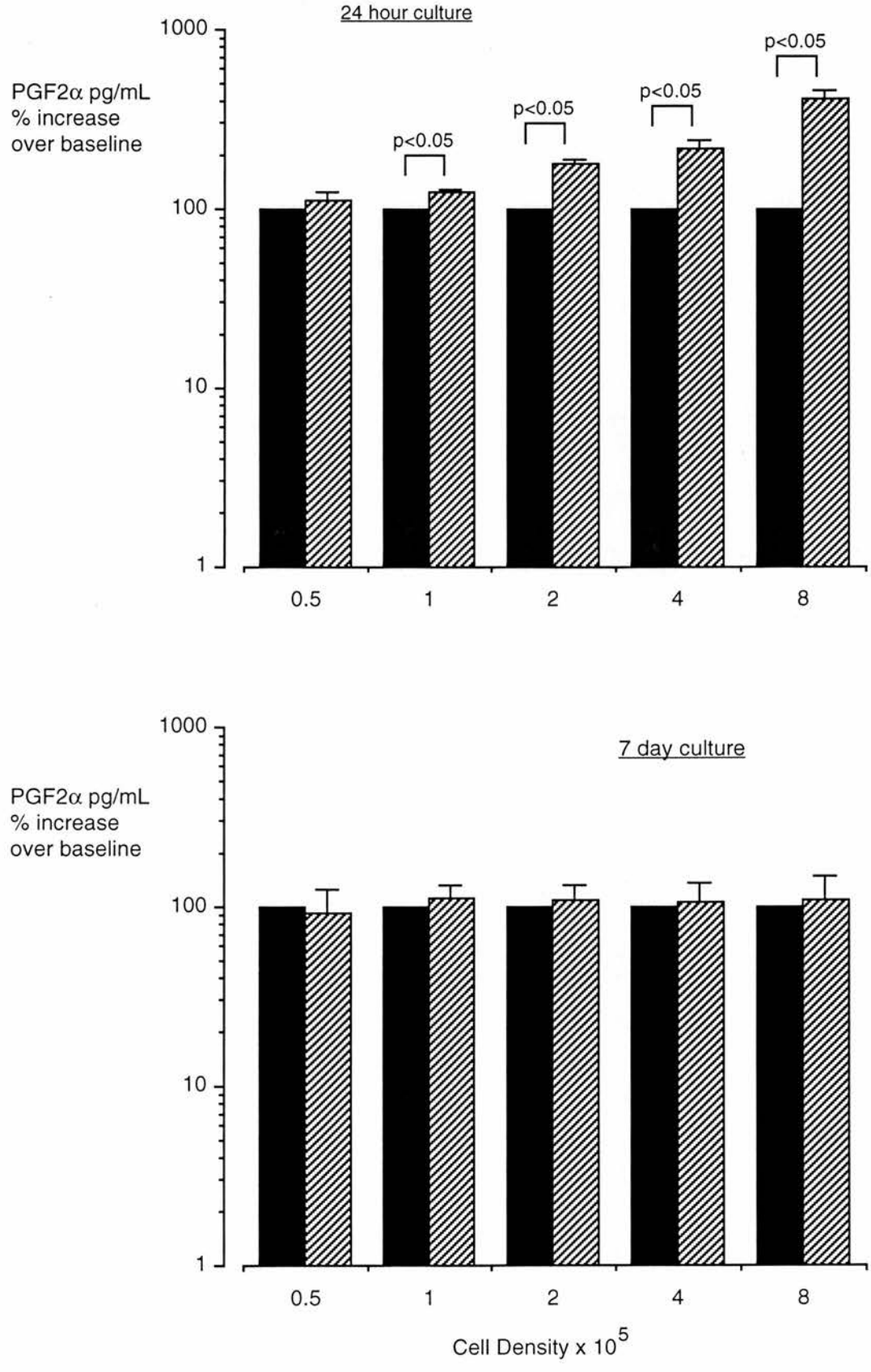

24 hour culture
PGF2α pg/mL
% increase
over baseline
1000
100
10
1
p<0.05
p<0.05
p<0.05
p<0.05
0.5
1
2
4
8
7 day culture
PGF2α pg/mL
% increase
over baseline
1000
100
10
1
0.5
1
2
4
8
Cell Density x 10^5

Figure 3.4

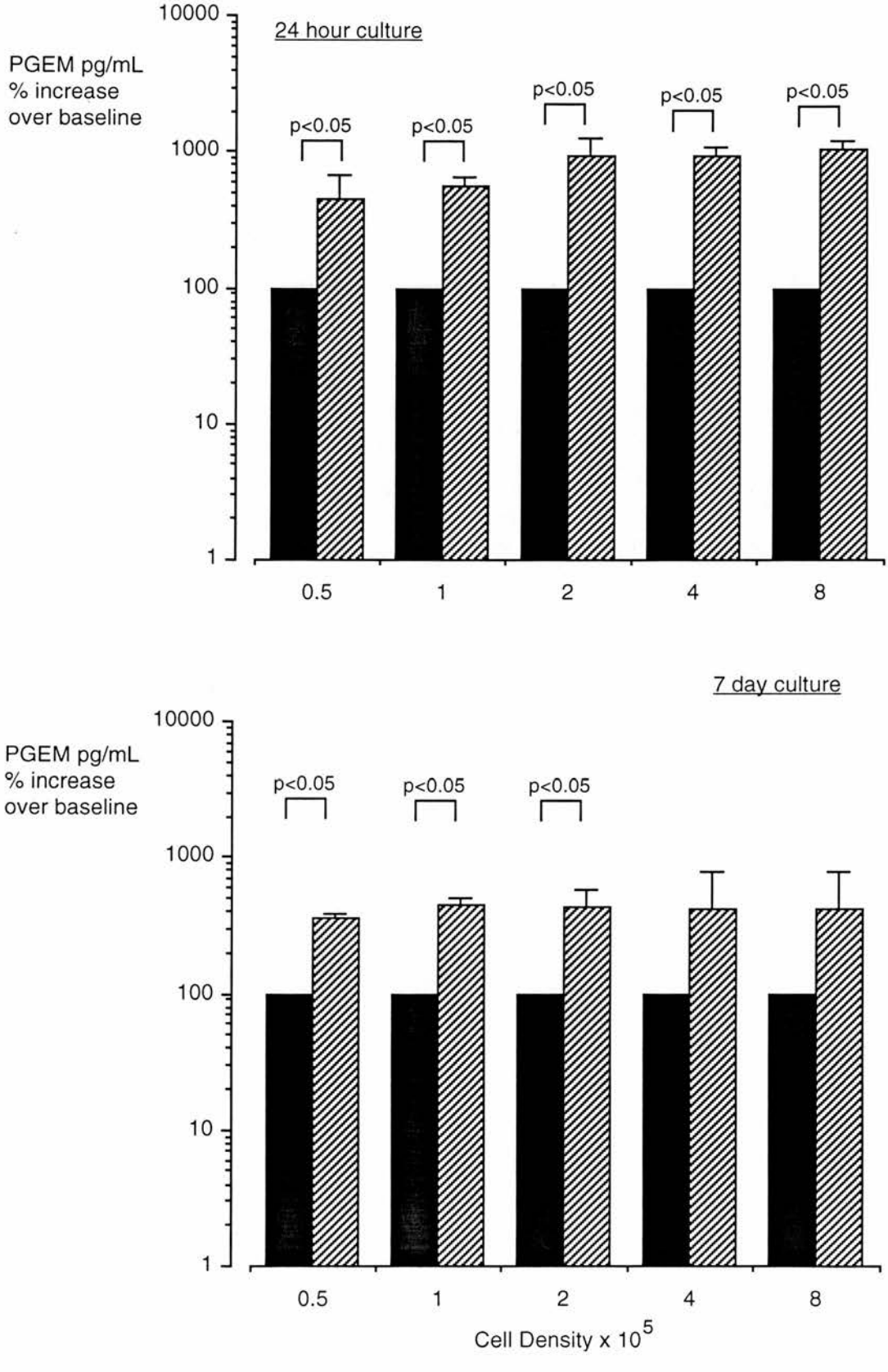

24 hour culture
PGEM pg/mL
% increase
over baseline
10000
1000
100
10
1
p<0.05
p<0.05
p<0.05
p<0.05
p<0.05
0.5
1
2
4
8
7 day culture
PGEM pg/mL
% increase
over baseline
10000
1000
100
10
1
p<0.05
p<0.05
p<0.05
0.5
1
2
4
8
Cell Density x 10^5

Figure 3.5

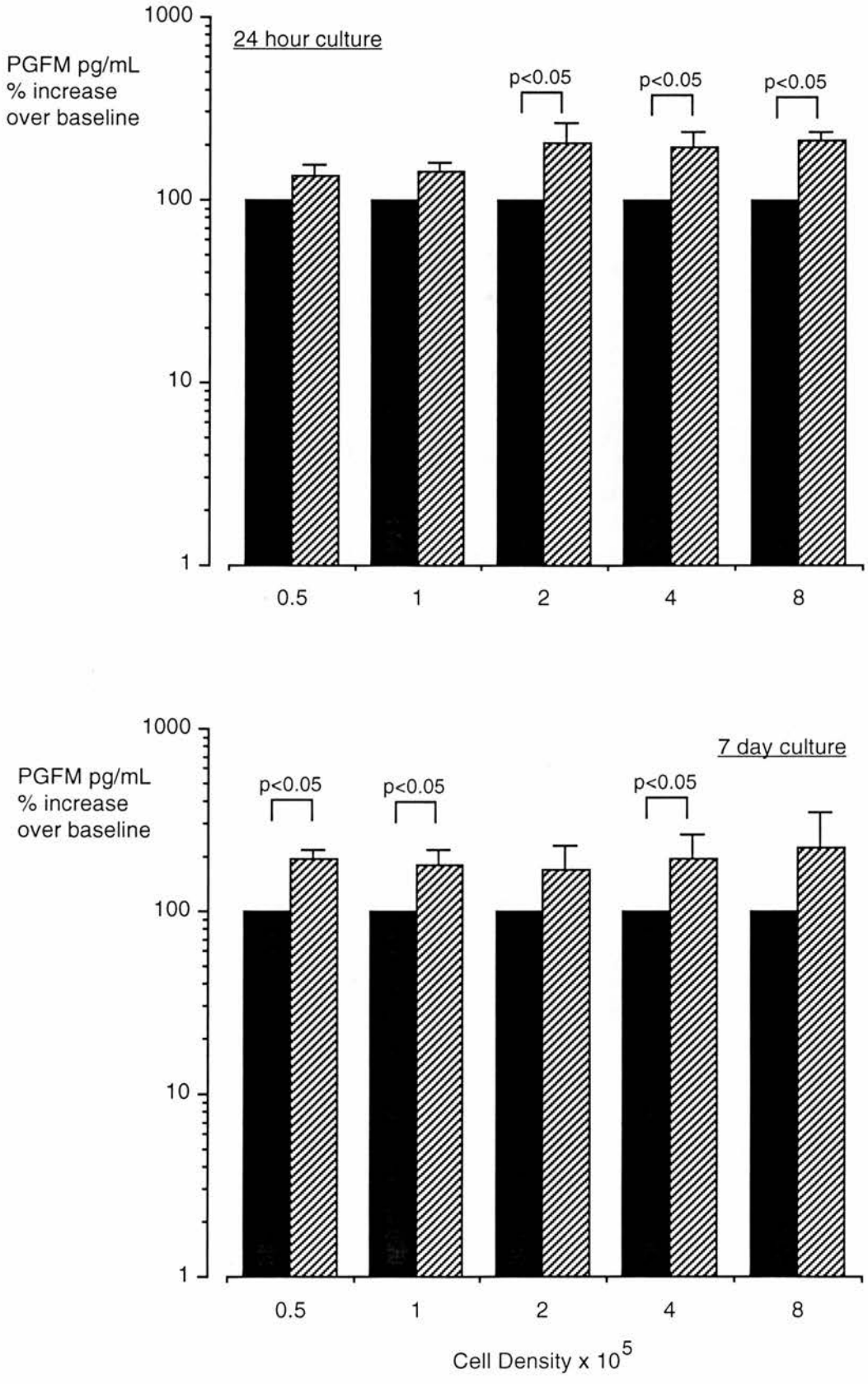
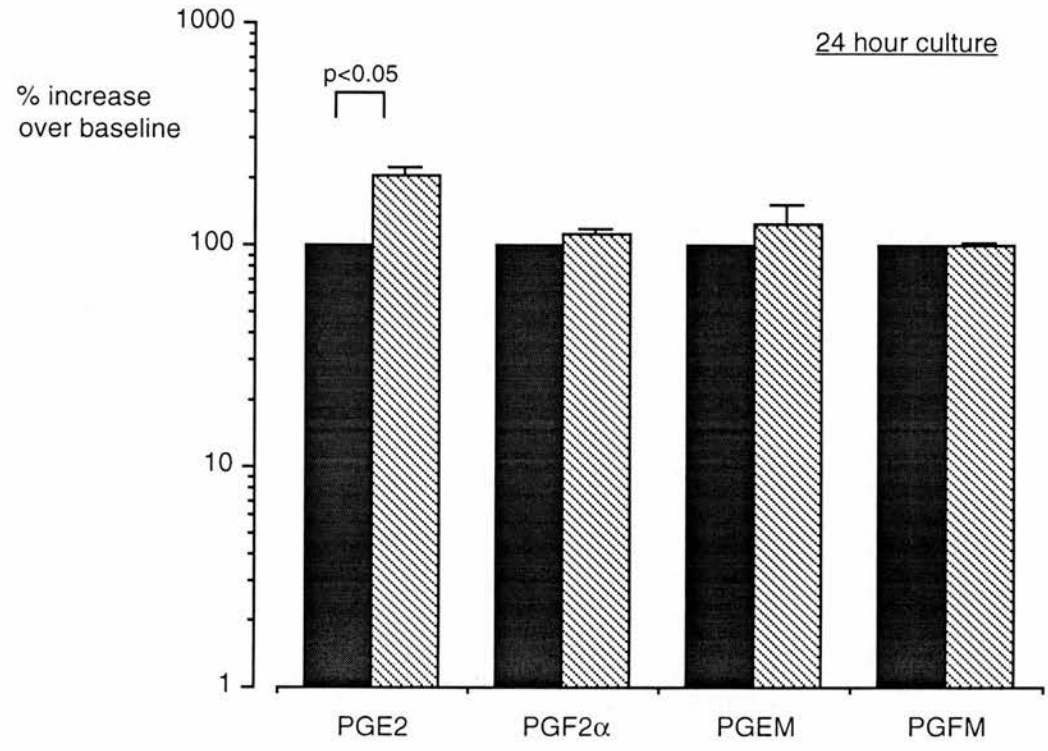

24 hour culture
PGFM pg/mL
% increase
over baseline
1000
100
10
1
p<0.05
p<0.05
p<0.05
0.5
1
2
4
8
7 day culture
PGFM pg/mL
% increase
over baseline
1000
100
10
1
p<0.05
p<0.05
p<0.05
0.5
1
2
4
8
Cell Density x 10^5

Figure 3.6


24 hour culture
p<0.05
% increase
over baseline
1000
100
10
1
PGE2
PGF2α
PGEM
PGFM

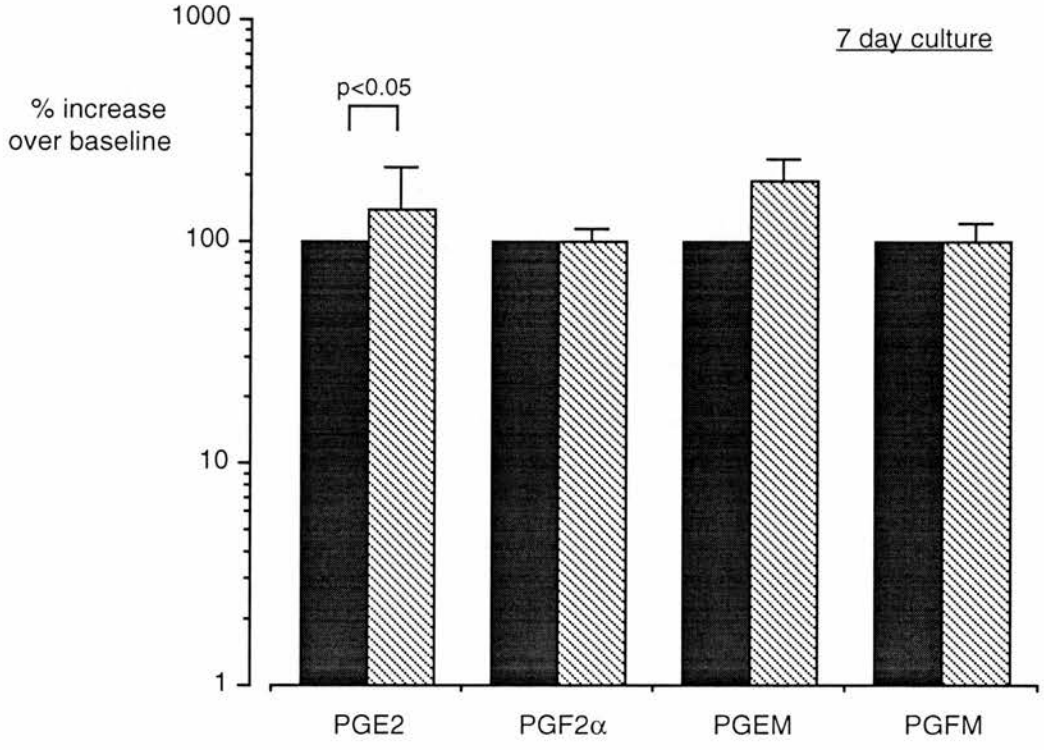

7 day culture
p<0.05
% increase
over baseline
1000
100
10
1
PGE2
PGF2α
PGEM
PGFM

Figure 3.7

Discussion.

These findings suggest that in amnion PKC-dependent mechanisms lose their responsiveness with increasing duration of culture. PKC can increase prostaglandin production via two pathways. Firstly, through its stimulatory effect on phospholipase A_2, PKC can increase the release of substrate arachidonic acid from membrane phosphatidylethanolamine. Secondly, PKC is able to stimulate transcription and translation, resulting in *de novo* synthesis of COX. Although there was no difference in basal production of prostaglandins between the 24 hour and seven day cultures, the capacity to increase production, either by substrate release or new enzyme synthesis is obviously lost in these amnion cultures. In the chorion cultures some response to PKC was maintained after seven days, possibly reflecting different metabolic properties of this cell type.

TNFα is a member of the cytokine family which acts non-enzymatically to regulate cellular function. The biological actions of cytokines are mediated via cell surface receptors and second messengers. A number of cytokines, including IL-1 and TNFα, have been shown to stimulate prostaglandin production by human amnion cells (Romero et al, 1989d; Lundin-Schiller and Mitchell, 1991). We have demonstrated increased PGE_2 production by amnion cells suggesting that receptors of TNFα are present at both culture time points. There was no response of chorion cells to TNFα after 24 hours in culture which may reflect a loss of receptors by this layer, perhaps as a result of trypsin digestion. There was some, albeit non-signifcant, stimulation of PGEM production after seven days in culture suggesting some recovery of receptor function.

Conclusions.

This methodology has confirmed that production of PGE_2 and $PGF_{2\alpha}$, and their respective metabolites is taking place at both 24 hours and seven days of culture. Although PGE_2 and PGFM production was less in the seven day cultures compared with 24 hours, and PGEM production was higher after seven days in culture, there was no significant difference in production between the two culture time periods, unlike the findings of other investigators (Gibb and Lavoie, 1990; Lundin-Schiller et al, 1991).

However, there were changes in the way the cultures responded to the phorbol ester PMA with increasing culture duration. By seven days of culture, responsiveness of amnion cells to PMA had been lost, and the chorion cells were not as uniformly responsive at seven days as they had been after 24 hours in culture. In view of the importance of protein kinase C (PKC) in substrate release and COX synthesis, and hence prostaglandin production, the experiments described in chapters four and five were therefore carried out in the first 48 hours of culture to avoid any loss of effect of PMA, as seen in the longer cultures. Chapter six describes experiments specifically investigating the effect of PKC inhibition on prostaglandin production, further illustrating the importance of employing a short term ($\leq$48 hour) culture period.

The increased PGE_2 production seen in response to TNFα suggests that the digestion process with trypsin has not damaged amnion receptors. Although we have not specifically investigated any receptor-mediated effects in the experiments described in chapters five and six, we have suggested that cytokines could be potential mediators of the stimulatory effect of prostaglandin production by amnion.

A cell density of 2 $x10^5$ /mL was employed throughout the culture experiments described in chapters five and six. Prostaglandin production at higher cell densities

was not significantly different for 2 $x10^5$ /mL, with the exception of PGE_2 at 8 $x10^5$ /mL. This cell density was chosen as practical and is in keeping with the cell culture work of other investigators in this field (Jones et al, 1989; Riley et al, 1992).

3.3. Response of Explant Cultures to Phorbol Myristoyl Acetate.

Methods.

Fetal membranes were collected at elective caesarean section (n=3) and amnion and chorion explant cultures prepared as described in section 2.3. All discs were suspended in 900 μL of culture medium. The discs were treated as follows: overnight incubation (approx. 24 hours) with 100 nM PMA; overnight incubation with no additions (control); overnight incubation followed by the addition of 100 nM PMA and further incubation for 24 hours; overnight incubation with no additions and further 24 hour incubation (control). The plates were incubated in humidified 95% air; 5% CO_2 at 37 C, the final culture volume was 1 mL, and all experiments were performed in duplicate. 0.5 mL of culture medium was aspirated from each well and oximated as described in section 2.5, pending radioimmunoassay (section 2.6).

Statistical Analysis.

Statistical analysis was performed using ANOVA. Data that were not normally distributed were log transformed prior to analysis.

Results.

PMA stimulated prostaglandin production by the amnion explants. The increase in PGE_2 production failed to reach significance in the explants treated in the first 24

hours of culture, but was significant in the explants treated in the second 24 hours of culture (Fig. 3.8; $p<0.005$). Stimulation of $PGF_{2\alpha}$ production by PMA was significant at both time points (Fig. 3.8; $p<0.001$). PGEM production by chorion, reflecting PGE_2, was also stimulated by PMA treatment at both time intervals (Fig. 3.9; $p<0.05$), whereas the phorbol ester had no effect on PGFM production by chorion explants (Fig. 3.9).

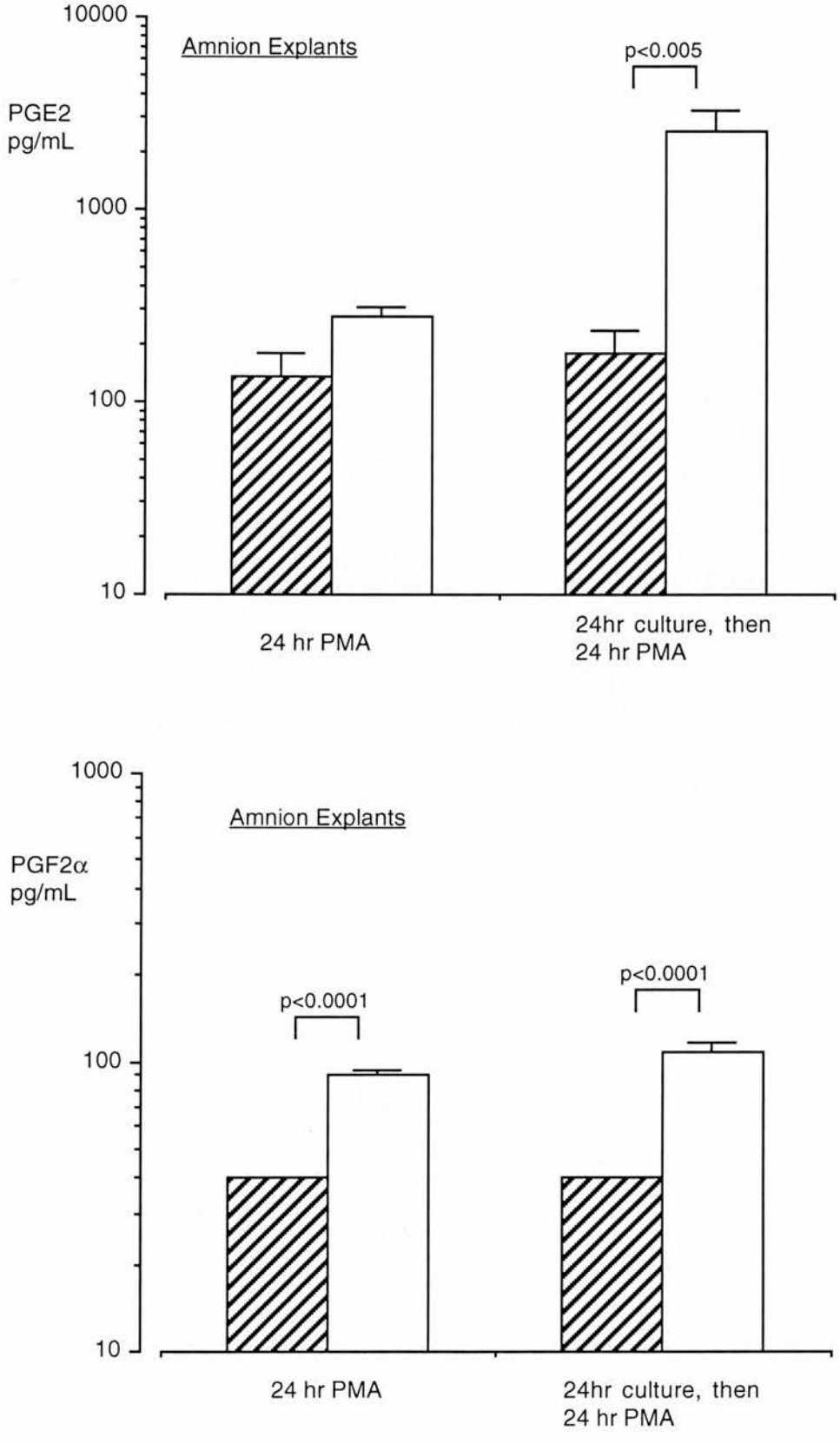
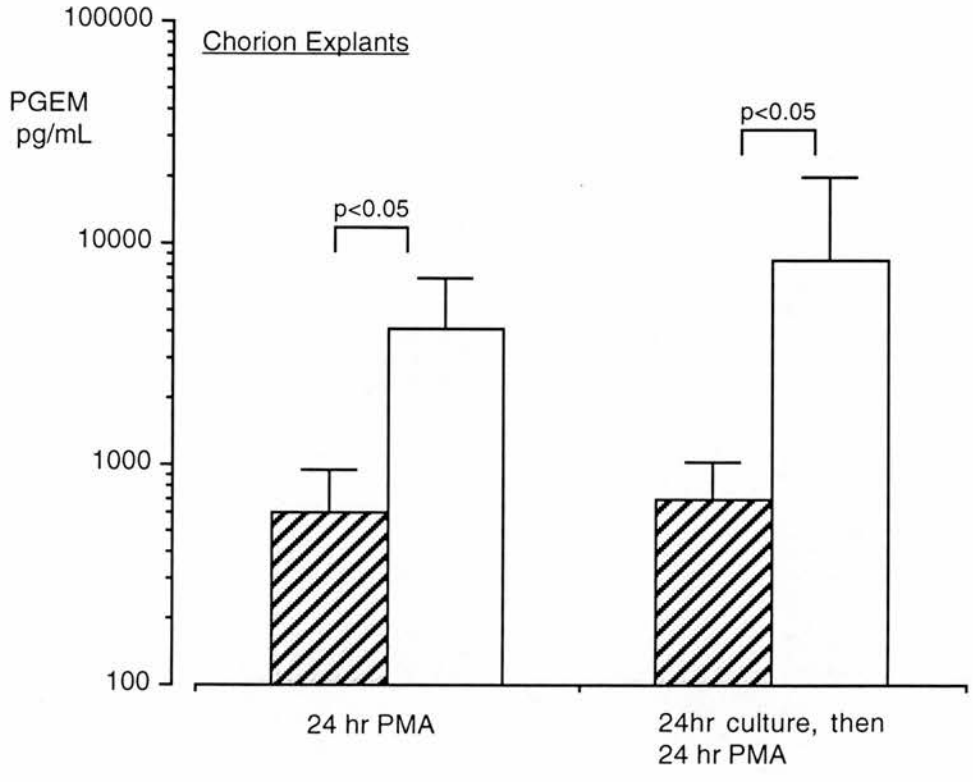

10000
1000
100
10
PGE2
pg/mL
Amnion Explants
p<0.005
24 hr PMA
24hr culture, then
24 hr PMA
1000
100
10
PGF2α
pg/mL
Amnion Explants
p<0.0001
p<0.0001
24 hr PMA
24hr culture, then
24 hr PMA

Figure 3.8


100000
Chorion Explants
PGEM
pg/mL
p<0.05
p<0.05
10000
1000
100
24 hr PMA
24hr culture, then
24 hr PMA

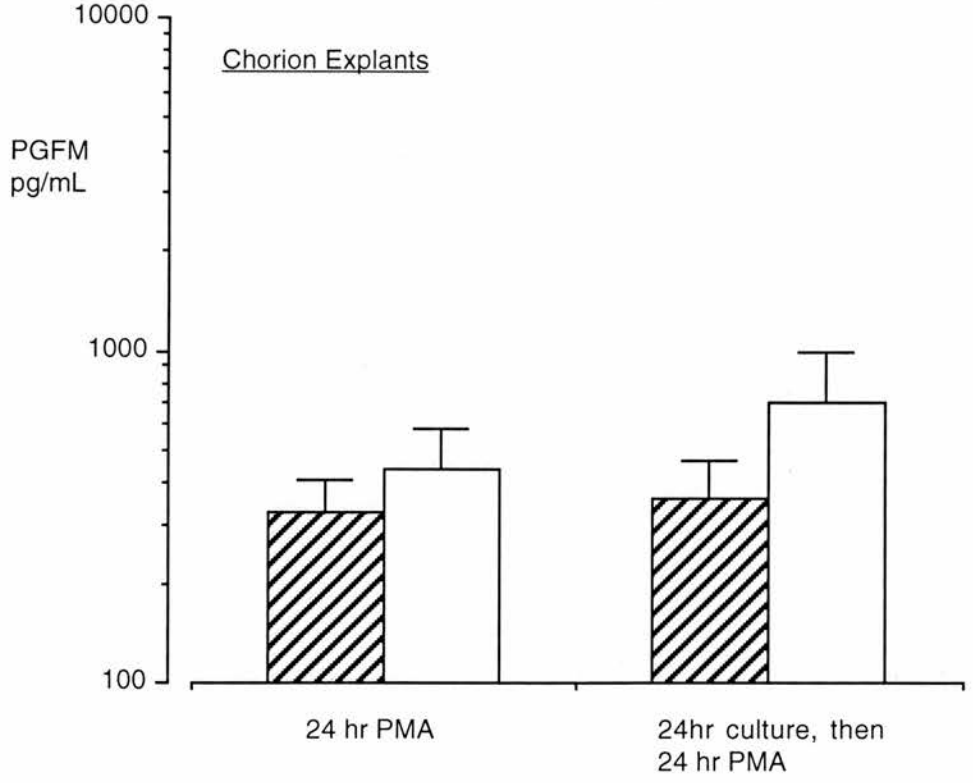

10000
Chorion Explants
PGFM
pg/mL
1000
100
24 hr PMA
24hr culture, then
24 hr PMA

Figure 3.9

Discussion.

The above experiments confirm, that using the explant culture technique described, production of prostaglandins E_2 and $F_{2\alpha}$, and their respective metabolites, by elective caesarean section tissue can be recorded. The response of these cultures to the phorbol ester PMA has demonstrated that protein kinase C-dependent mechanisms are functioning in these cultures. As discussed in section 3.2 PKC is an important mediator of prostaglandin production via its promotion of substrate release (arachidonic acid), and activation of COX. The experiments described in chapter four investigate changes in prostaglandin synthesis and metabolism in fetal membranes collected after elective caesarean section and spontaneous labour. In these experiments we investigated the effect of exogenous substrate, PMA, and the COX inhibitor indomethacin on prostaglandin production. In order to look at any effect that parturition might have on synthesis and metabolism we elected to carry out these experiments in the first 24 hours of culture, accepting that in the above experiments the stimulatory effect of PMA with regard to PGE_2 production just failed to reach significance. However, as described in the experiments in chapter four, PMA significantly stimulated PGE_2 production in both groups of tissue in the first 24 hours of culture.

Chapter Four

Changes in Prostaglandin Synthesis and Metabolism Associated with Labour, and the Influence of Dexamethasone, RU 486 and Progesterone.

Introduction.

Prostaglandins are intimately involved in the process of parturition. This is evidenced by the increase in concentrations of PGE_2, $PGF_{2\alpha}$ and their metabolites in maternal plasma (Green et al, 1974; Lackritz et al, 1978, Satoh et al, 1979), urine and amniotic fluid (Satoh et al, 1979; Keirse et al, 1977a) at the onset of spontaneous labour. The fetal membranes appear to be the source of the increase in prostaglandins seen with labour. The amnion produces PGE_2 and has little metabolising activity (Okazaki et al, 1981a) whereas the chorion is the major site of prostaglandin metabolism, being rich in prostaglandin dehydrogenase (Okazaki et al, 1981a; Cheung et al, 1989; Cheung et al, 1990). This enzyme converts PGE_2 and $PGF_{2\alpha}$ to their respective metabolites 13,14-dihydro-15-keto-PGE_2 (PGEM) and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM). There are two mechanisms by which this increase in prostaglandin levels can be effected: firstly, an increase in their synthesis, or secondly, a decrease in their metabolism.

It is well established that prostaglandin production by amnion is increased in spontaneous labour (Cheung et al, 1989; Skinner and Challis, 1985; Reddi et al, 1990). However, in order to exert their uterotonic effect on the myometrium, prostaglandins synthesised by the amnion would have to escape the metabolic activity of prostaglandin dehdrogenase within the chorion, and there are conflicting reports as to whether or not this is possible. Some reports have confirmed that transfer of prostaglandins across full-thickness membranes does occur, with the majority of the prostaglandin remaining intact (Bennett et al, 1990; Nakla et al, 1986), whereas others have shown that transfer does not take place (McCoshen et al, 1990). Any reduction in the metabolising capacity of the chorion could potentially enhance prostaglandin transfer. There is little information available on changes in prostaglandin metabolism

associated with labour, with one report suggesting that there is no difference in the metabolising capacity of pre- and post- labour tissues (Cheung et al, 1989).

Although the effect of steroid hormones on prostaglandin production has been examined extensively (Mitchell et al, 1988a; Potestio et al, 1988), this is not the case for prostaglandin metabolism and no previous study has examined prostaglandin metabolism in parallel. It has been shown that antiprogestagenic steroids alter prostaglandin metabolism in the guinea-pig (Kelly and Bukman, 1990), but there is little information on the effect of progesterone and corticosteroids on prostaglandin metabolism in chorion.

The aim of this study was to assess, in parallel, the alterations in prostaglandin synthesis and metabolism in amnion and chorion respectively that are associated with spontaneous labour and to investigate the effect of steroid hormones on prostaglandin metabolism.

Subjects and Methods.

Fetal membranes were obtained from women at term with uncomplicated pregnancies. Group one laboured spontaneously and achieved a vaginal delivery without oxytocin augmentation (n=12), and group two underwent elective caesarean section for either breech presentation or previous caesarean section (n=12).

Amnion and chorion explant cultures were prepared as described in section 2.3.1. The explant discs of amnion were treated with the following (final concentrations): 100μM arachidonic acid (Sigma, UK); 100nM phorbol myristoyl acetate (PMA) (Sigma, UK); 2.8mM indomethacin (Sigma, UK) or complete culture medium, the latter to act as

control. The solutions of arachidonic acid, PMA and indomethacin were made up in complete culture medium (section 2.4.2). The volume added was 100μL and the discs were therefore cultured in a total fluid volume of 1mL. All experiments (n=12 in each group) were performed in duplicate (ie. 2 discs per experiment per patient giving 8 discs in total per patient).

The plates were incubated for 18 hours in humidified 5% CO_2 in air at 37 C. Following incubation, 0.5mL of incubation medium was aspirated from each well and oximated with an equal volume of methyl oximating solution. These samples were thoroughly mixed, stored at room temperature for 24 hours and then at 5 C pending radioimmunoassay.

The chorion explant discs were treated with one of the following (final concentrations): 1mM dexamethasone, 1mM progesterone (both Sigma, UK), 1mM RU 486 (Roussel Laboratories) or complete culture medium alone to act as control. The explants were incubated for 18 hours in humidified 5% CO_2 in air at 5 C.

The following solutions were added (final concentrations): 100mM arachidonic acid; 500ng PGE_2 (Upjohn Ltd, UK); 500ng $PGF_{2\alpha}$ (Upjohn Ltd, UK) and complete culture medium, the latter to act as control. The total volume of culture fluid was maintained at 1mL. All experiments (n=12 in each group) were performed in duplicate (ie. 2 discs per experiment per patient giving 8 discs in total per patient). The explants were incubated for a further 4 hours and then 0.5mL of incubation medium aspirated from each well for oximation as described in section 2.5.

Radioimmunoassay .

The competitive binding radioimmunoassay employed in these experiments is described in detail in section 2.6.

Statistical Analysis.

Analysis of variance (ANOVA) was used to analyse the data. Where the data were not normally distributed, log transformation was employed prior to ANOVA.

Results.

The Effect of Labour on Prostaglandin Synthesis by Amnion and Chorion (Fig. 4.1).

Basal production of PGE_2 and $PGF_{2\alpha}$ was significantly higher in amnion obtained following spontaneous labour compared with elective caesarean section ($p<0.05$). PGEM and PGFM production did not differ significantly between spontaneous labour and caesarean section tissue. $PGF_{2\alpha}$ production was substantially less than that of PGE_2.

PGEM was the major product of the chorion in both spontaneous labour and caesarean section groups, and production was significantly higher in the spontaneous labour tissue ($p<0.05$). PGFM was produced in smaller amounts and only minimal levels of PGE_2 and $PGF_{2\alpha}$ were recorded. There was no significant difference in the production of either primary prostaglandin or PGFM between the two groups.

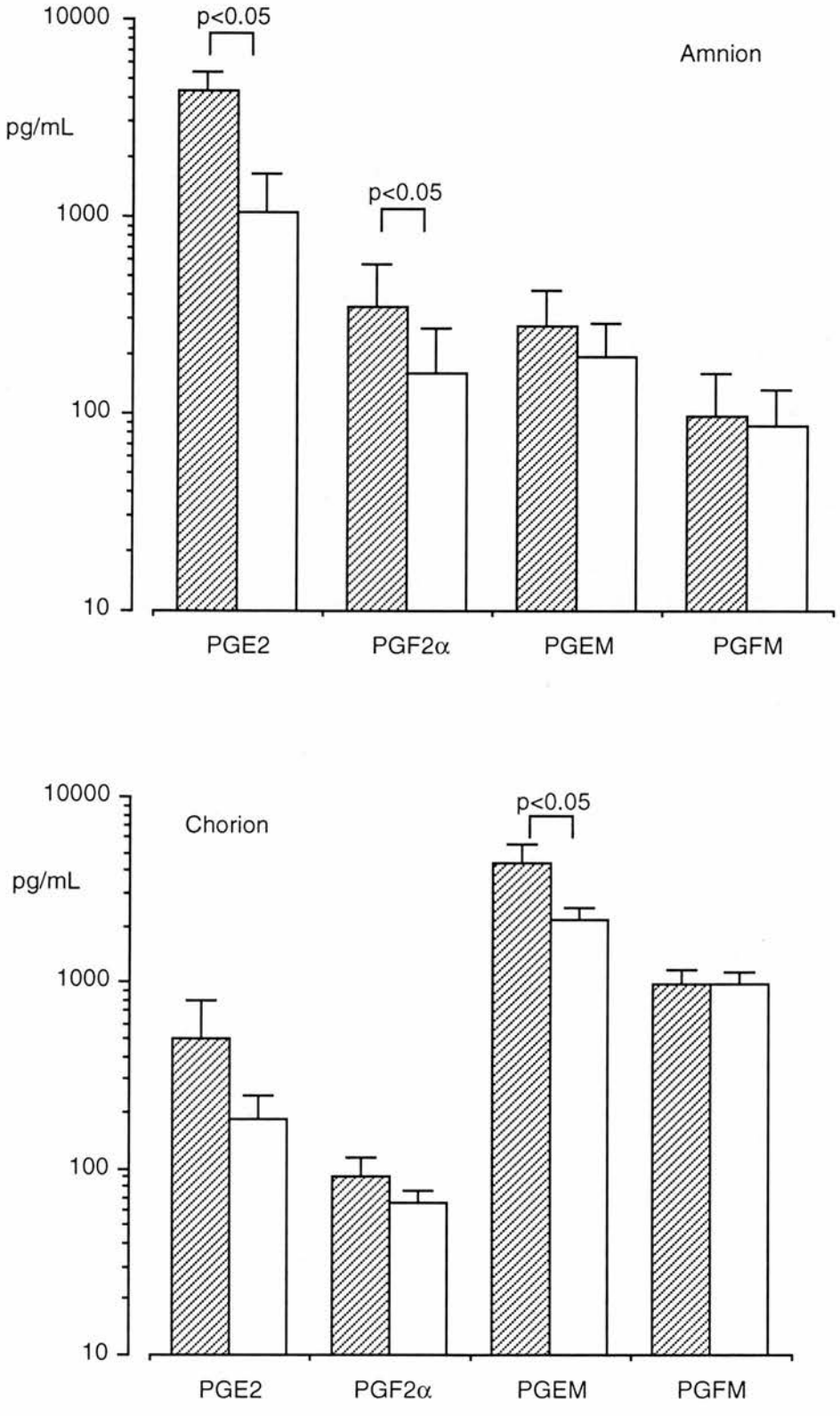

10000
1000
100
10
pg/mL
p<0.05
p<0.05
Amnion
PGE2
PGF2α
PGEM
PGFM
10000
1000
100
10
pg/mL
Chorion
p<0.05
PGE2
PGF2α
PGEM
PGFM

Figure 4.1

The Effect of Arachidonic Acid, Phorbol Myristoyl Acetate and Indomethacin on Prostaglandin Synthesis in Amnion (Fig. 4.2).

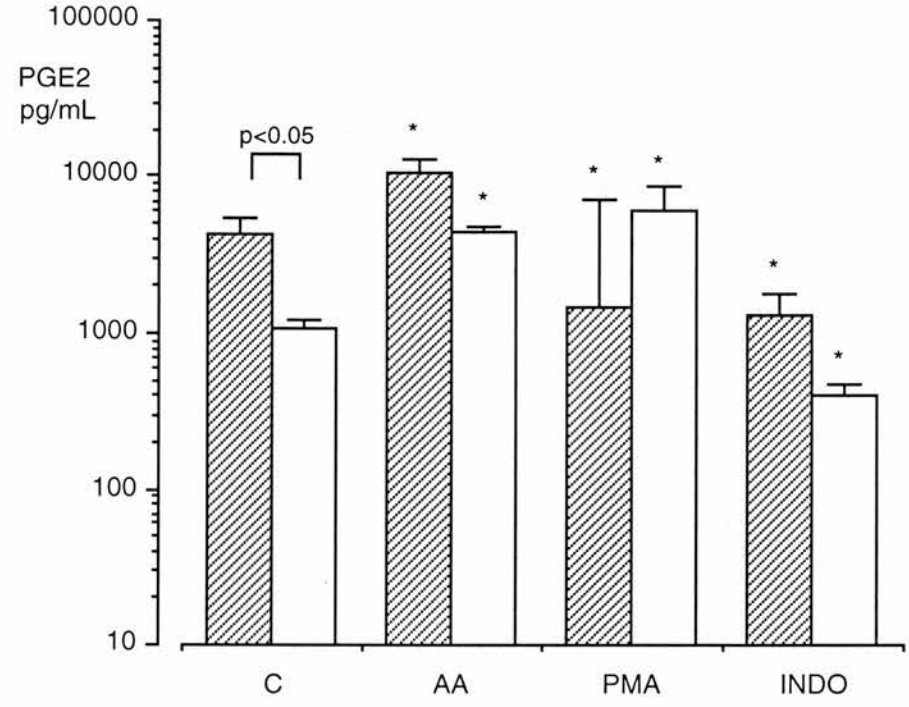
The addition of arachidonic acid resulted in significant stimulation of PGE_2 and $PGF_{2\alpha}$ synthesis by amnion obtained following both spontaneous labour and caesarean section in comparison with the basal synthesis by the two groups ($p<0.05$) (Note: PGE_2 expressed in ng/ml; $PGF_{2\alpha}$ in pg/ml). The percentage increase in prostaglandin production over basal levels was greater in the caesarean section tissue and this was significant for $PGF_{2\alpha}$ ($p<0.01$). PMA stimulated PGE_2 production from amnion in both groups ($p<0.05$). There was no significant stimulation of $PGF_{2\alpha}$ production following the addition of PMA in either group. Indomethacin inhibited PGE_2 production in both groups ($p<0.05$). Also, $PGF_{2\alpha}$ production was inhibited by indomethacin in amnion obtained following spontaneous labour ($p<0.05$).

The Effect of Labour on Prostaglandin Metabolism in Chorion (Fig. 4.3):

Metabolism of exogenous PGE_2 to PGEM was the same by chorion from both groups. Similarly, there was no difference in the metabolism of exogenous $PGF_{2\alpha}$ to PGFM between the groups.

The Effect of Steroids on Prostaglandin Metabolism in Chorion (Fig. 4.4):

Dexamethasone and progesterone had no effect on prostaglandin metabolism. However, RU 486 decreased the metabolism of added PGE_2 by chorion obtained following spontaneous labour but not by elective section tissue.


100000
PGE2
pg/mL
p<0.05
10000
1000
100
10
C
AA
PMA
INDO

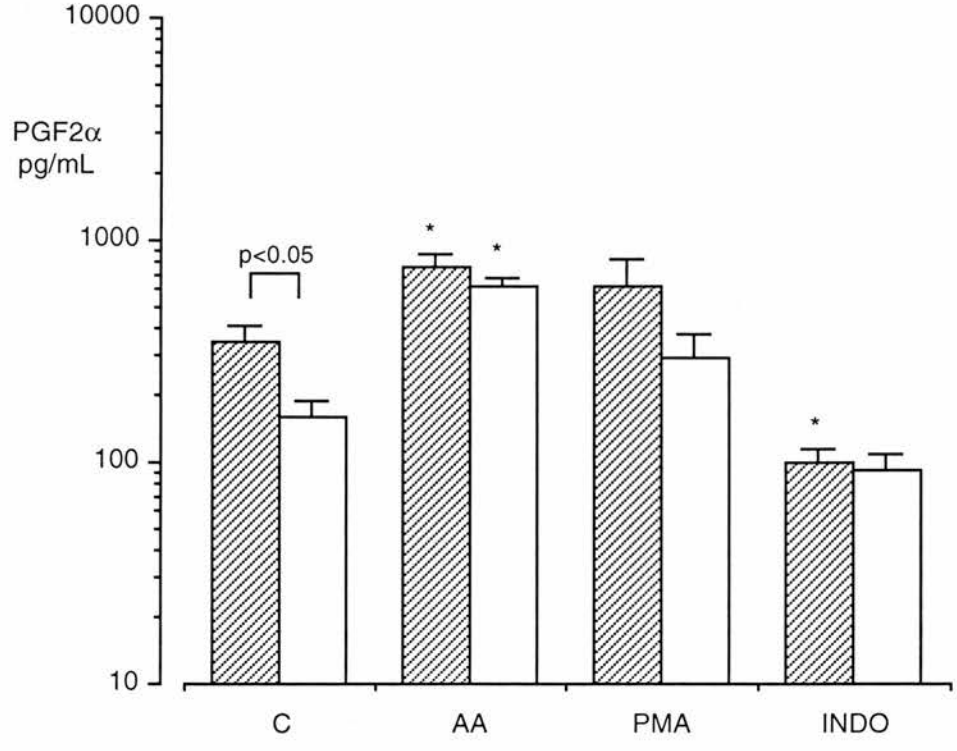

10000
PGF2α
pg/mL
1000
p<0.05
100
10
C
AA
PMA
INDO

Figure 4.2

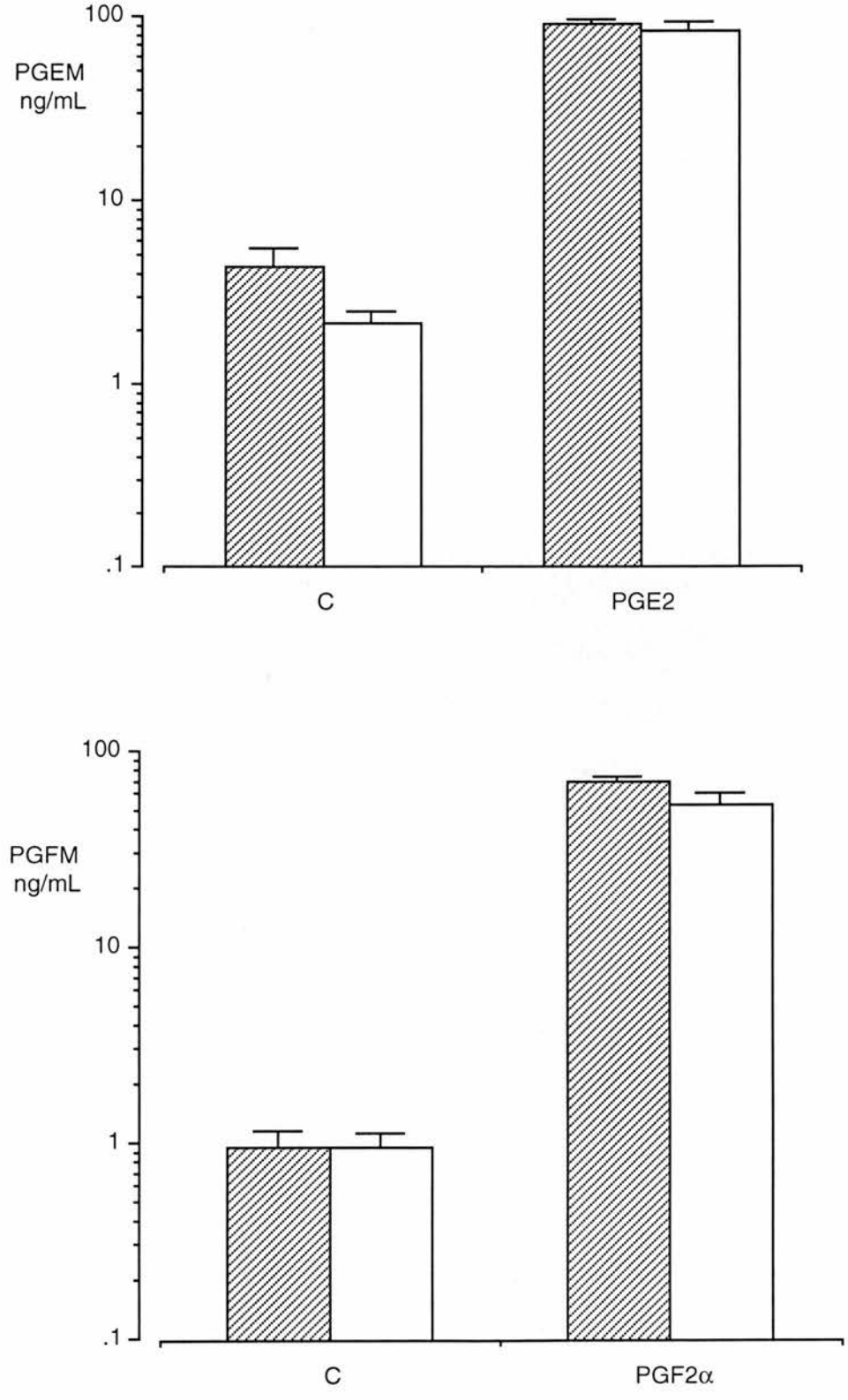


Figure 4.3

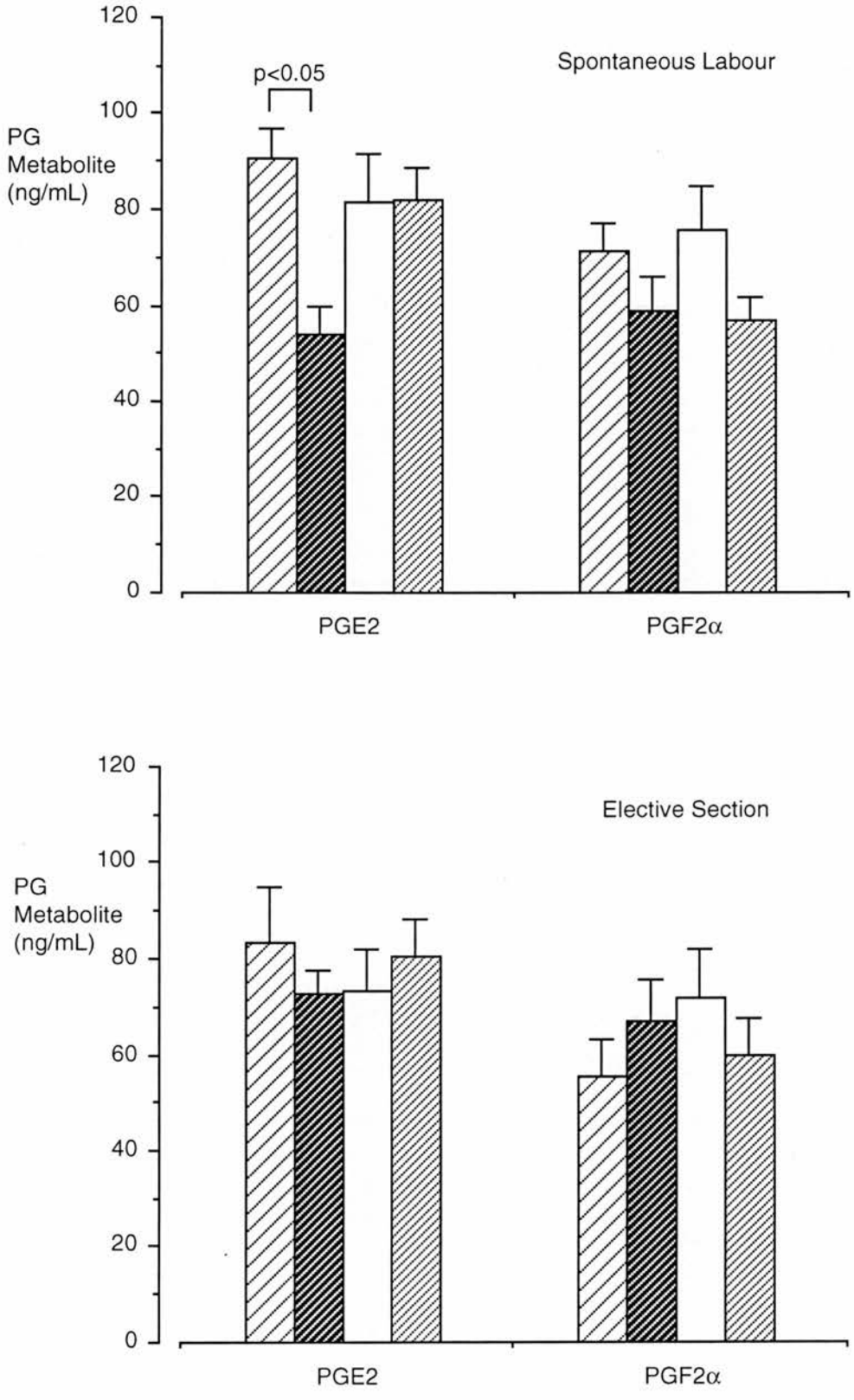

120
100
80
60
40
20
0
PG
Metabolite
(ng/mL)
p<0.05
Spontaneous Labour
PGE2
PGF2α
120
100
80
60
40
20
0
PG
Metabolite
(ng/mL)
Elective Section
PGE2
PGF2α

Figure 4.4

Discussion.

The aim of this study was to compare changes in prostaglandin synthesis and metabolism in parallel by looking at both the amnion and chorion collected from each subject. The major product of the cultured amnion explants was PGE_2 which is in keeping with reports from others (Okazaki et al, 1981a; Mitchell et al, 1978c). We were able to demonstrate a significant increase in PGE_2 production by amnion obtained following spontaneous labour, in keeping with the findings of previous studies (Cheung et al, 1989; Skinner et al, 1985; Reddi et al, 1990). Although measured in much smaller concentrations, we also found a significant increase in the production of $PGF_{2\alpha}$ by the amnion following spontaneous labour which could not be explained by any cross-reactivity as PGE_2 cross-reactivity with $PGF_{2\alpha}$ assay was <0.02%. Okazaki *et al.* (1981a) were unable to demonstrate $PGF_{2\alpha}$ production by the amnion. However, Mitchell *et al.* (1978c) detected small amounts of $PGF_{2\alpha}$ in their superfusates of amnion tissue. It is established that the main intrauterine source of $PGF_{2\alpha}$ is the decidua and any contribution that the amnion makes to increased $PGF_{2\alpha}$ concentrations is likely to be negligible. Indeed, the presence of $PGF_{2\alpha}$ in the culture system could be explained by non-enzymatic conversion from PGE_2, or by production by fibroblasts or macrophages present within the deeper layers of the amnion. Production of both PGEM and PGFM by the amnion was minimal, reflecting the virtual absence of prostaglandin dehydrogenase activity in this tissue (Cheung et al, 1990).

The production of PGE_2 was decreased in both groups when the amnion explants were incubated with the cyclooxygenase inhibitor indomethacin. This finding confirms that the PGE_2 measured has been synthesised *de novo* in the culture system rather than being residual prostaglandin remaining within the tissue. Similarly, $PGF_{2\alpha}$ production was inhibited in the spontaneous labour group. There was no

significant inhibition in the caesarean section group, however basal concentrations were very low suggesting that minimal production was occurring, and therefore an inhibitory agent would have little effect. This resultant decrease in prostaglandin synthesis by the amnion underlines the rationale for prescribing indomethacin in the management of preterm labour.

In our experiment, the addition of arachidonic acid resulted in significant stimulation of PGE_2 and $PGF_{2\alpha}$ production by amnion obtained both before and after labour compared to basal concentrations. Arachidonic acid is the obligatory precursor of prostaglandins of the 2 series and related compounds. It has been shown that there is an increase in the arachidonic acid concentration of amniotic fluid during labour (MacDonald et al, 1974), and in particular that the increase in arachidonic acid concentration is disproportionately high compared to other free fatty acids. The mobilisation of arachidonic acid from membrane glycerophospholipids is thought to be the rate-limiting step in prostaglandin production, and this involves the enzyme phosholipase A_2. An increase in the activity of this enzyme with advancing gestation has been described (Okazaki et al, 1981c), but an increase in activity associated with the onset of labour was not demonstrated. The significant difference between the two groups in basal production of both primary prostaglandins was lost following the addition of arachidonic acid substrate. These findings suggest that the availability of arachidonic acid is a limiting factor in prostaglandin production by the elective section tissue.

The difference in basal production of PGE_2 and $PGF_{2\alpha}$ between the groups could be explained by a difference in the activity of cyclooxygenase (COX) in pre- and post-labour amnion. Cyclooxygenase (COX) is responsible for the conversion of arachidonic acid to the cyclic endoperoxides which are in turn converted to the primary prostaglandins. Lopez Bernal *et al.* (1987) compared PGE_2 production by

spontaneous preterm and term amnion cells and showed that the prostaglandin output of term cells remained significantly greater than the preterm cells in the presence of arachidonic acid, suggesting a maturation in the activity of COX toward term. Further results supporting this theory come from Olson *et al.* (1991) who found a bimodal distribution of COX activity in amnion collected at elective caesarean section implying that there is maturation of the enzyme system prior to the onset of labour. Indeed, increased expression of the cyclooxygenase gene has been demonstrated in human fetal membranes and placenta with the onset of labour (Bennett et al, 1992b). However, in our experiment the significant difference in prostaglandin production between the groups was not maintained following the addition of arachidonic acid. If there was increased cyclooxygenase activity in the spontaneous labour tissue we would expect this difference in prostaglandin production to remain. In fact, the increase in production of both PGE_2 and $PGF_{2\alpha}$ over basal levels appears to be greater in caesarean section compared with spontaneous labour tissue, and this was statistically significant for $PGF_{2\alpha}$. This finding suggests that, provided adequate substrate is available, both groups have similar capacities for prostaglandin production. However, the effect of exogenous arachidonic acid on cyclooxygenase activity is not known, and it is possible that the arachidonic acid may have induced and saturated the enzymes thus abolishing any differences seen previously.

The addition of PMA resulted in increased production of PGE_2 by amnion in both groups compared with control values. Phorbol esters, such as PMA and tumor promoter phorbol ester (TPA), act by stimulating the activity of the enzyme protein kinase-C (PKC), a calcium- and lipid-dependent protein kinase which is involved in the regulation of many cellular functions. PKC activity has been identified in human amnion and decidua vera tissues (Okazaki et al, 1984) and it is thought to be involved in arachidonic acid release and the activation of COX (Zakar and Olson, 1988). There is evidence to support the existence of multiple isoforms of COX (Xie et al, 1992;

Kujubu et al, 1991; O'Banion et al, 1991). COX-1 is the constitutive form of the enzyme, whereas COX-2 can be induced by a number of agents including phorbol esters. Previous reports have shown that tumor promoter phorbol ester significantly increases PGE_2 production when added to cultured human amnion cells (Zakar and Olson, 1988). This study of amnion explants confirmed the stimulatory effect of phorbol ester on PGE_2 production. We did not find a similar effect with regard to $PGF_{2\alpha}$ production, but this may simply be related to the lower levels of this prostaglandin being synthesised by the tissue in comparison to PGE_2 (347 v 4364 pg/ml). The response of the amnion cultures to PMA was variable, particularly in the spontaneous labour tissues, a finding that has also been noted in previous work using TPA (Zakar and Olson, 1992). Reports have shown that phorbol ester treatment can enhance PGE_2 production by amnion cells isolated both before (Sander and Myatt, 1990) and after (Zakar and Olson, 1988) labour. There was no significant difference in response to PMA between the spontaneous labour and caesarean section amnions in our experiment . These results suggest that COX activity is the same in both groups. One explanation for this is that COX-2 activity has been maximally induced by PMA and any original difference in COX activity has been lost.

Significant PGEM production occurred in the chorion, which is likely to reflect PGE_2 production because of the metabolising capacity of the chorion for prostaglandins via 15-hydroxy-prostaglandin dehydrogenase (PGDH), the predominant enzyme in this tissue (Okazaki et al, 1981a). PGFM, reflecting $PGF_{2\alpha}$ production, was substantially less than PGE_2/PGEM. These results are in accordance with Okazaki *et al.* (1981a). We found no difference in the metabolism of added PGE_2 and $PGF_{2\alpha}$ to their respective metabolites confirming the report of Cheung and Challis (1989) who demonstrated no change in the metabolism of PGE_2 added to cell cultures of chorion obtained pre- and post-labour. This is compatible with the finding that PGDH localisation in the placenta and membranes is not altered in association with labour

(Cheung et al, 1990), although it has been demonstrated that PGDH mRNA in chorio-decidua is reduced in assocation with spontaneous labour (Sangha et al, 1994). It therefore appears that the increase in prostaglandin concentrations associated with spontaneous labour are not the result of a decrease in metabolic activity within the chorion.

Mifepristone (RU 486) is a well established medical abortifacient and has more recently been successfully employed as an agent for labour induction at term (Frydman et al, 1992). *In vitro* studies exploring the effect of RU 486 on endometrial (Kelly et al, 1986b) and early decidual cells (Smith and Kelly, 1987) have shown that prostaglandin synthesis is stimulated, and metabolism inhibited by the antiprogestin. In addition, work in the guinea-pig has shown decreased prostaglandin metabolism in myometrium and chorion following *in vivo* treatment with RU 486 (Kelly and Bukman, 1990). In our experiment, there was no difference in the basal production of prostaglandin metabolites by the chorion explants following in vitro treatment with RU 486. This was the same for both spontaneous labour and elective section tissue. However, the metabolism of exogenous PGE_2 to PGEM was significantly reduced in the spontaneous labour tissue following RU 486 treatment. A similar trend was seen in the metabolism of exogenous $PGF_{2\alpha}$ in the spontaneous labour group, although this did not reach significance. There was no decrease in the metabolism of either prostaglandin by elective section tissue. These findings point to a reduction in the activity or amount of prostaglandin dehydrogenase in the tissue following treatment with RU 486. Recent work on decidua obtained from women pre-treated with RU 486 in early pregnancy has shown, by both a direct enzymatic method and immunohistochemistry, that the activity of PGDH is reduced in this tissue compared with control (Cheng et al, 1993). This decrease in the metabolic capacity of the chorion following treatment with RU 486 would reduce the threshold for prostaglandin synthesis necessary to generate uterine activity and could explain the

clinical effects of this agent with regard to labour induction. It was surprising that the reduction in PGE_2 metabolism following treatment with RU 486 was confined to spontaneous labour tissue and was not found in caesarean section tissue. Although basal levels were comparable there may still be a lower degree of prostaglandin dehydrogenase activity in tissues obtained following spontaneous labour which may not be evident under basal conditions in the culture system. When RU486 is added an additive or synergistic effect may result, so explaining the results seen in the experiments on chorion taken from women in spontaneous labour. Such a lower degree of prostaglandin metabolism within the tissue with RU 486 may be in keeping with previous *in vivo* studies (Cheng et al, 1993). The increase in prostaglandin concentrations at the level of the fetal membranes which would result from such inhibition of metabolism could contribute to the enhanced uterine activity seen both in spontaneous labour and following treatment with RU486. Clearly an endogenous tissue factor associated with labour is required as the effect was not seen with tissues treated with RU 486 taken from women not in labour. It is also possible that the difference between changes in metabolism seen following *in vivo* treatment compared with that following *in vitro* treatment with RU 486 may reflect an intermediate step in this process.

Dexamethasone has been shown to inhibit prostaglandin production from placental and amnion cell cultures (Riley et al, 1992). This occurred in a dose dependent manner and the addition of arachidonic acid to the placental cell cultures did not abolish the inhibitory effect of dexamethasone suggesting that this steroid is acting at the level of COX activity. An alternative mechanism, resulting in decreased prostaglandin concentrations, would be an increase in PGDH activity with an associated increase in prostaglandin metabolism. An increase in the tissue activity of PGDH in rat lung and kidney following treatment with prednisolone has been reported (Moore and Hoult, 1980). However, we were unable to demonstrate increased prostaglandin metabolism

in response to dexamethasone in our chorion explants. Similarly, Gibb *et al* (1988) were unable to demonstrate an increase in prostaglandin metabolism to account for the reduction in PGE_2 output by their chorion cells incubated in the presence of dexamethasone.

In the sheep, progesterone withdrawal is a prerequisite of the onset of labour. In humans, there is no decrease in circulating systemic progesterone concentrations prior to the onset of spontaneous labour. However, systemic steroid concentrations may not reflect local changes in steroid environment within the fetal membranes which may control the process of parturition in a paracrine fashion. In our experiment the addition of progesterone had no effect on prostaglandin metabolism. This is in contrast with the inhibitory effect of RU 486. One possible explanation for this is that endogenous progesterone is exerting tonic control over prostaglandin metabolism which could not therefore be altered by additional progesterone, but could be overcome by the antiprogestin.

In summary, this study has shown that prostaglandin synthesis by fetal membranes is increased in association with parturition, but that there is no alteration in prostaglandin metabolism by chorion in association with labour. However, metabolism in spontaneous labour tissue can be inhibited by the addition of RU 486.

Chapter Five

The Influence of Amniotic Fluid on Prostaglandin Synthesis and Metabolism in Human Fetal Membranes.

Introduction.

As previously discussed, prostaglandins are thought by many investigators to be central to the process of parturition. Since amnion is principally involved in PGE_2 synthesis it has been extensively investigated as a potential regulator of prostaglandin dynamics in association with parturition.

Amnion is an avascular tissue which is in contact with both the maternal and fetal environment. Production of PGE_2 could therefore be regulated by a factor(s) present in amniotic fluid, the nature of which may be controlled by the fetus. Alternatively, the decidua, which is maternal in origin, or the chorion, may influence prostaglandin production by the amnion. Amniotic fluid contains stimulators and inhibitors of prostaglandin production. An endogenous inhibitor of prostaglandin synthase has been demonstrated in amniotic fluid (Saeed et al, 1982), the activity of which decreases with advancing gestation and the onset of spontaneous labour. This suggests that the amnion is under tonic inhibition which is gradually lost as pregnancy progresses. Further evidence supporting the role of inhibitory activities includes the finding that conditioned media from amnion obtained at caesarean section will inhibit endometrial cell prostaglandin output (Manzai and Liggins, 1984), and that amniotic fluid contains compounds that appear to inhibit phospholipase activity (Wilson et al, 1985). Stimulatory activities exist in amniotic fluid (Dowling et al, 1991; Cohen et al, 1985) and there is an increase in this activity with advancing gestation. There is therefore a reciprocal change in the stimulatory and inhibitory properties of amniotic fluid that is gestation-dependent (Cohen et al, 1985). Examples of agents that stimulate amnion cell prostaglandin production include epidermal growth factor (EGF), transforming growth factor α (TGFα) and platelet activating factor (PAF), all of which are present in amniotic fluid in increasing concentrations during labour (Mitchell, 1988b; Romero et al, 1989b).

The chorion is an important site of prostaglandin metabolism, and any change in metabolic activity within this tissue could significantly alter active prostaglandin concentrations. Since amniotic fluid contains factors which influence prostaglandin production by amnion, it is possible that these agents could also alter the metabolic activity of the chorion. As no previous studies have assessed the effect of amniotic fluid on production and metabolism simultaneously, the purpose of this study was to determine the effect of amniotic fluid on prostaglandin production and metabolism by the fetal membranes, amnion and chorion.

Subjects and Methods.

Amniotic fluid was collected from two groups of pregnant women at term (37-42 weeks) with an uncomplicated pregnancy. The first group laboured spontaneously and achieved a vaginal delivery without oxytocin augmentation (n=24); the second group was delivered by elective caesarean section for either breech presentation or previous caesarean section (n=24). Fetal membranes were collected from a third group of women undergoing uncomplicated elective caesarean section at term (n=4).

Cell Culture Preparation.

Amnion and chorion cell cultures, at a density of $2x10^5$ cells per well, were prepared as described in section 2.2.1. All cultures were maintained for seven days, at which time the cells remained at confluence and there was no evidence of bacterial contamination.

Effect of amniotic fluid on amnion and chorion prostaglandin production:

The plates of amnion and chorion were incubated in humidified 95% air; 5% CO_2 at 37 C for 24 hours. Amniotic fluid obtained at elective caesarean section (n=6) and following spontaneous labour (n=6) was added to the cells in volumes of 50, 100, 200 and 400 μL. Cells were also incubated in media alone, to act as control, and with phorbol myristoyl acetate 100nM (Sigma, UK) to confirm that prostaglandin synthesis could be induced in these preparations. The total volume of each well was maintained at 1 mL. Amniotic fluid (100μL) from each subject was also incubated alone for estimation of background primary prostaglandins and their metabolites. All experiments were performed in duplicate. The plates were incubated for a further 24 hours and then 0.5mL aspirated from each well for prostaglandin measurement. The above experiment was repeated on a second set of fetal membranes using amniotic fluid from different subjects (again, n=6 for both groups).

Amniotic fluid and chorion cell metabolism of exogenous prostaglandins:

Plates of chorion cells were incubated for 24 hours in humidified 95% air; 5% CO_2 at 37 C. Amniotic fluid collected following elective caesarean section (n=6) and spontaneous labour and delivery (n=6) was added to the cells and the plates incubated for a further 24 hours. Chorion cells and 400μL of each amniotic fluid sample were incubated alone to enable estimation of background levels of the primary prostaglandins and their metabolites. The following additions were made (final concentrations): 500ng PGE_2 (Upjohn Ltd, UK) and 500ng $PGF_{2\alpha}$ (Upjohn Ltd, UK). For the final four hour incubation the plates therefore comprised wells containing: chorion cells alone; cells+400μL amniotic fluid+500ng PGE_2; cells+400μL amniotic fluid+500ng $PGF_{2\alpha}$; cells+500ng PGE_2 (control); cells+500ng

$PGF_{2\alpha}$ (control); 400µL amniotic fluid alone. The final incubation volume in all wells was 1mL. All experiments were performed in duplicate. The incubation fluid (0.5mL) was oximated and stored pending radioimmunoassay. The experiment was repeated on a second set of chorion with different amniotic fluids (again, n=6 for each group).

Radioimmunoassay.

The competitive binding radioimmunoassay employed in these experiments is described in detail in section 2.6.

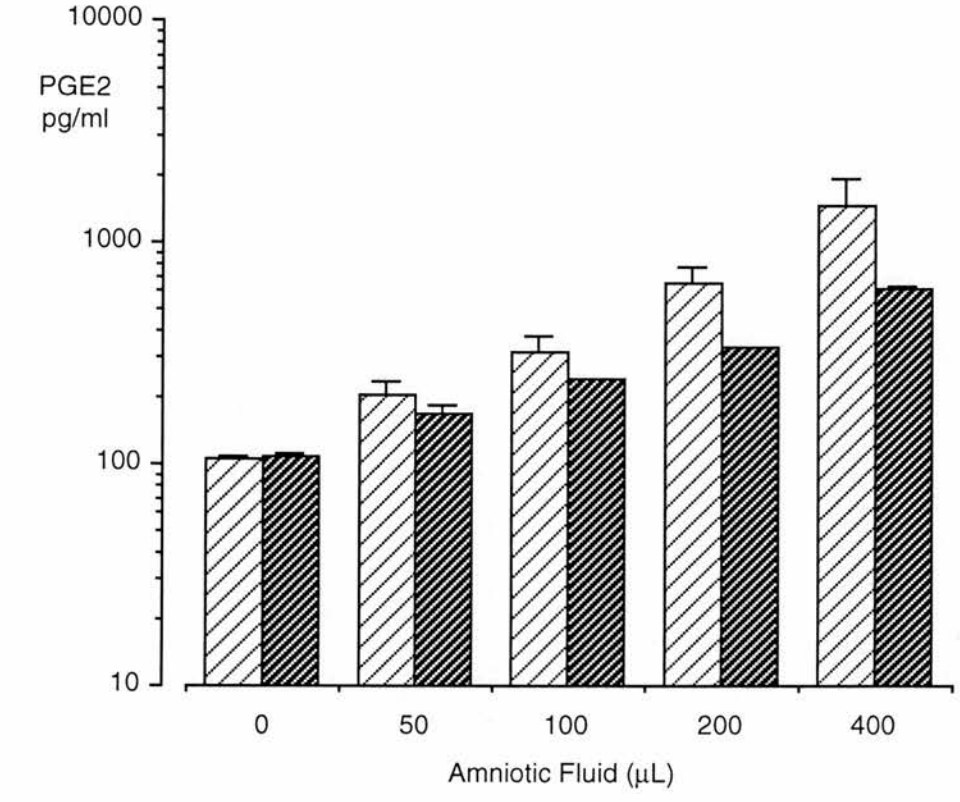
Statistical Analysis.

One and two factor analysis of variance (ANOVA) was used to analyse the data. Where the data were not normally distributed, log transformation was employed prior to ANOVA. Background concentrations of prostaglandins and their metabolites in the amniotic fluid were subtracted in all experiments prior to statistical analysis.

Results.

The effect of amniotic fluid on prostaglandin production by amnion and chorion:

The stimulatory effect of amniotic fluid, in a dose-dependent manner, on prostaglandin E_2 and prostaglandin $F_{2\alpha}$ production by amnion is illustrated in Figures 5.1 and 5.2 respectively. Amniotic fluid from spontaneous labour stimulated significantly greater production of both prostaglandins compared with elective caesarean section fluid: PGE_2, $p<0.001$; $PGF_{2\alpha}$, $p<0.05$. Amniotic fluid from spontaneous labour produced significantly more PGEM from chorion, in a dose-dependent manner, than caesarean section amniotic fluid, $p<0.0001$ (Fig. 5.3). This was not confirmed in the second culture. Production of PGFM by chorion cells, reflecting $PGF_{2\alpha}$, was significantly greater in the presence of spontaneous labour amniotic fluid, compared with elective section fluid, at all doses, $p< 0.003$ (Fig. 5.4).


10000
PGE2
pg/ml
1000
100
10
0
50
100
200
400
Amniotic Fluid (μL)

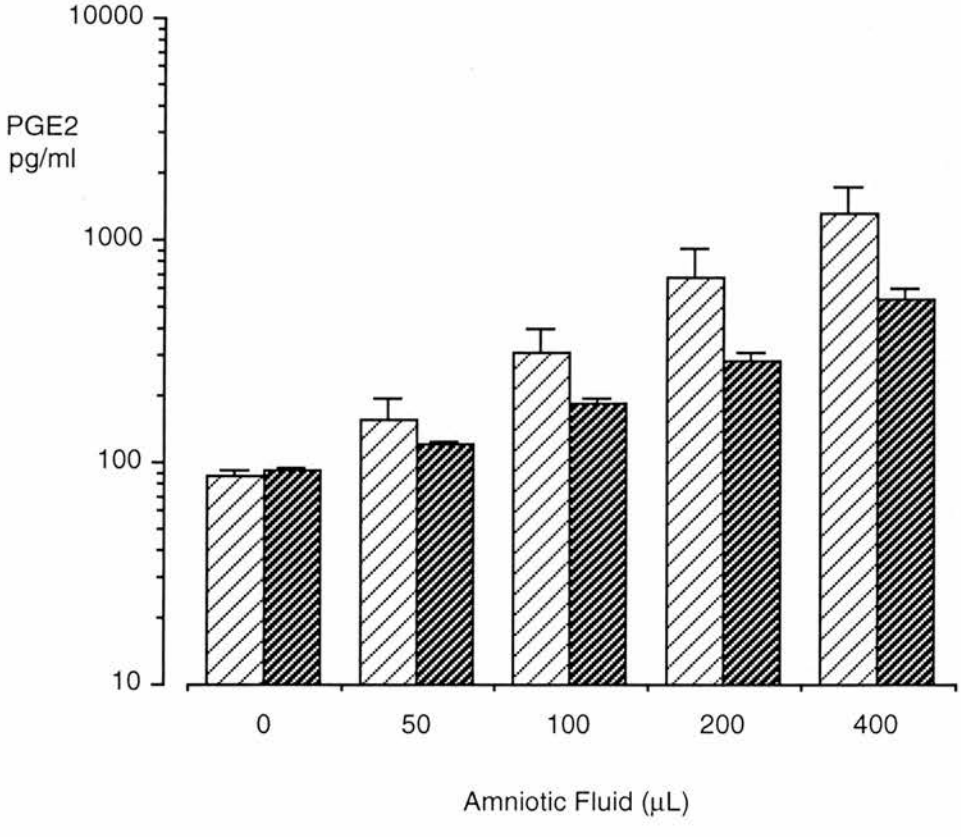

10000
PGE2
pg/ml
1000
100
10
0
50
100
200
400
Amniotic Fluid (μL)

Figure 5.1

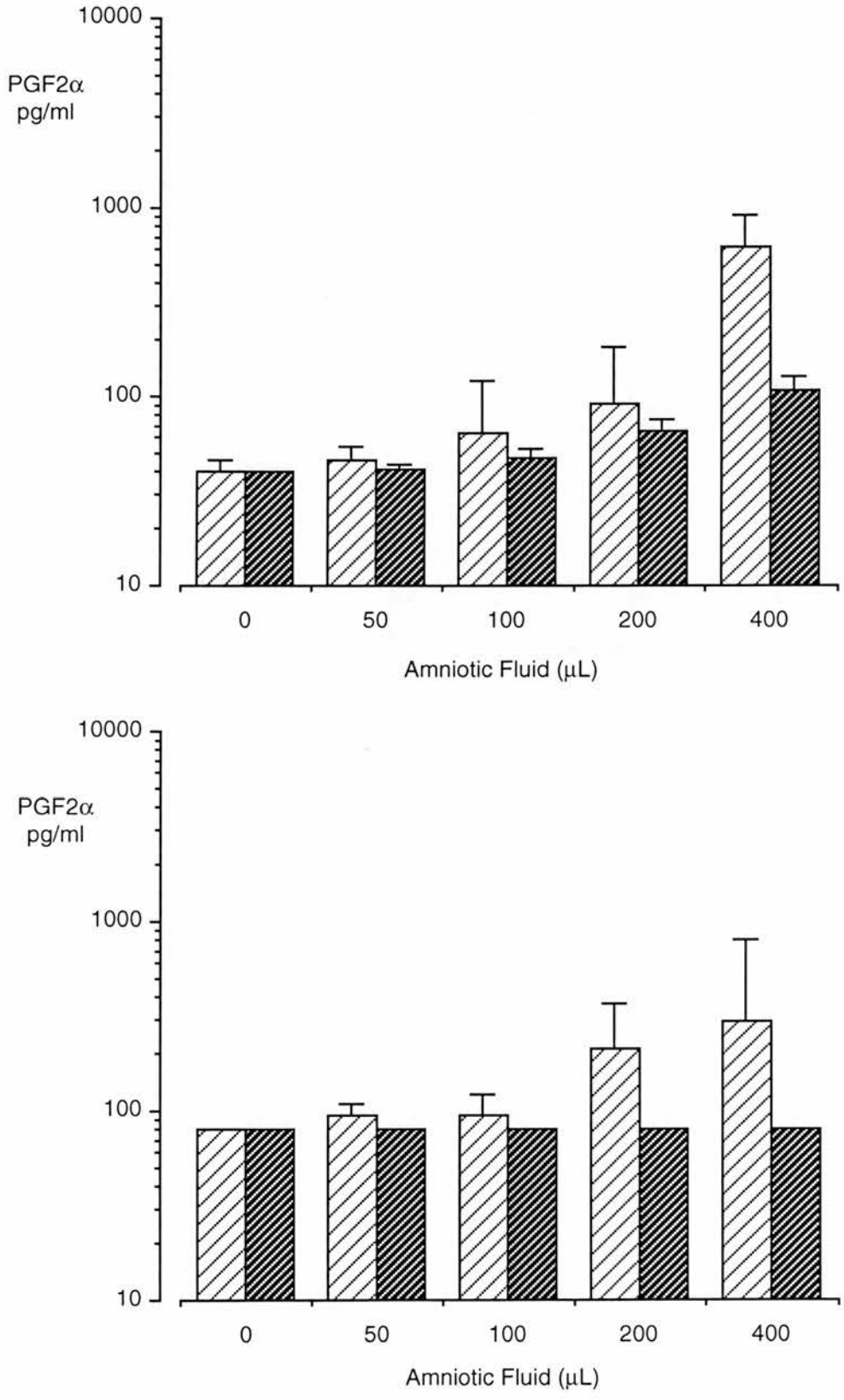
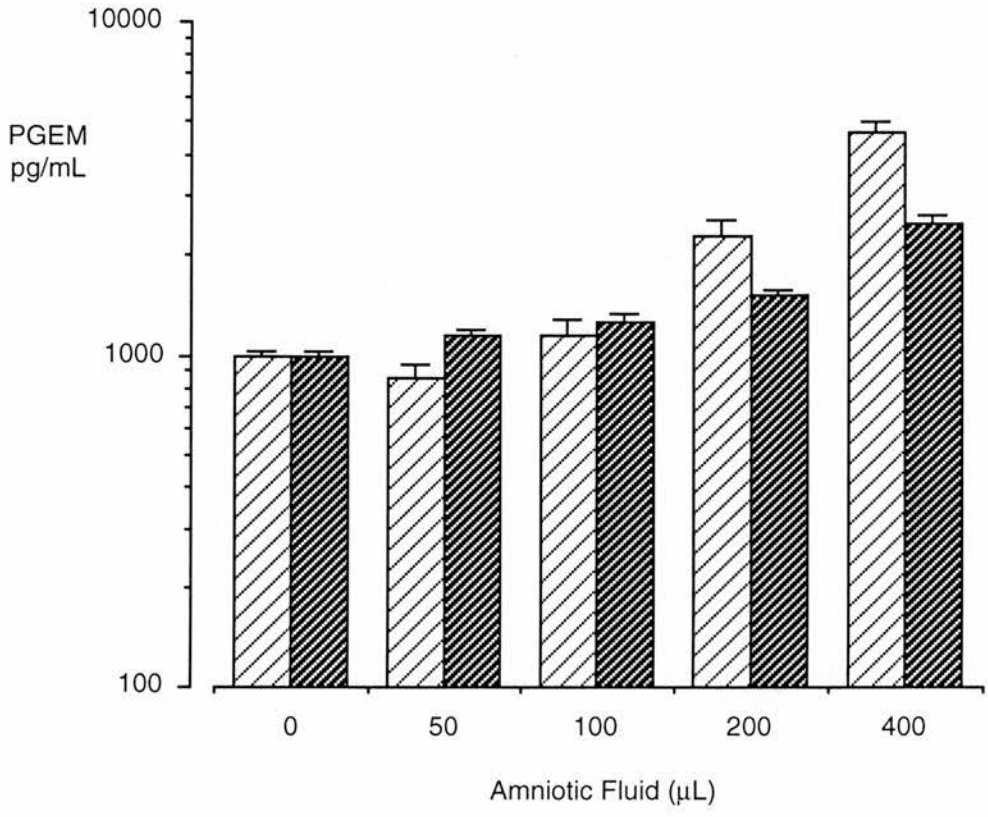
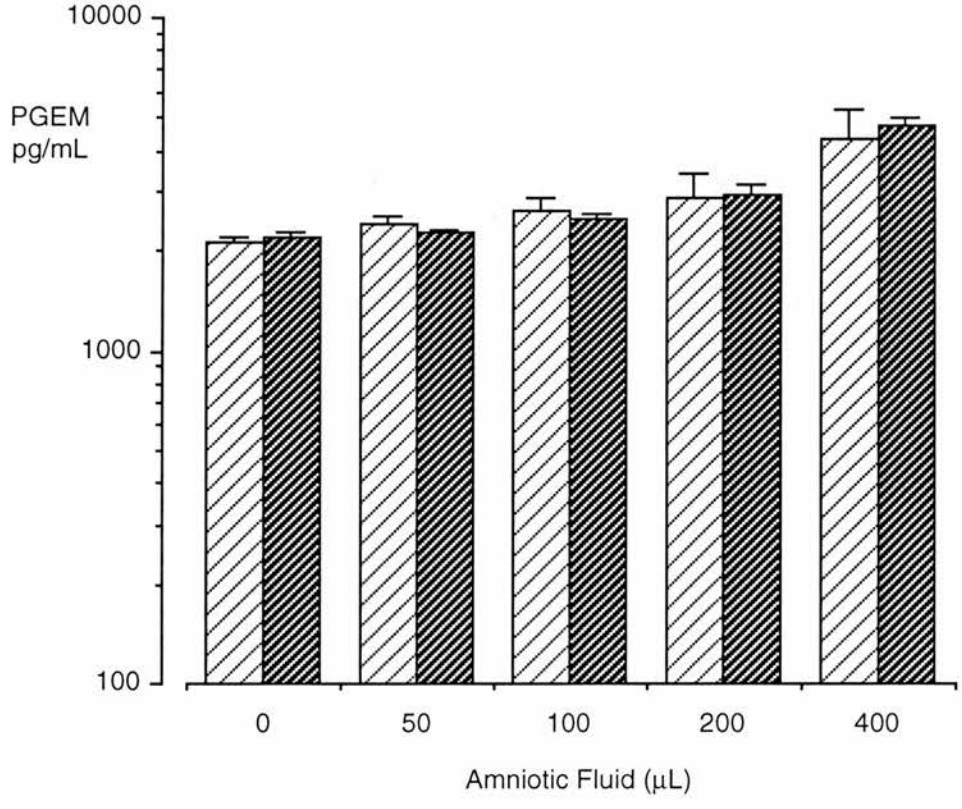

10000
PGF2α
pg/ml
1000
100
10
0
50
100
200
400
Amniotic Fluid (μL)
10000
PGF2α
pg/ml
1000
100
10
0
50
100
200
400
Amniotic Fluid (μL)

Figure 5.2


10000
PGEM
pg/mL
1000
100
0
50
100
200
400
Amniotic Fluid (µL)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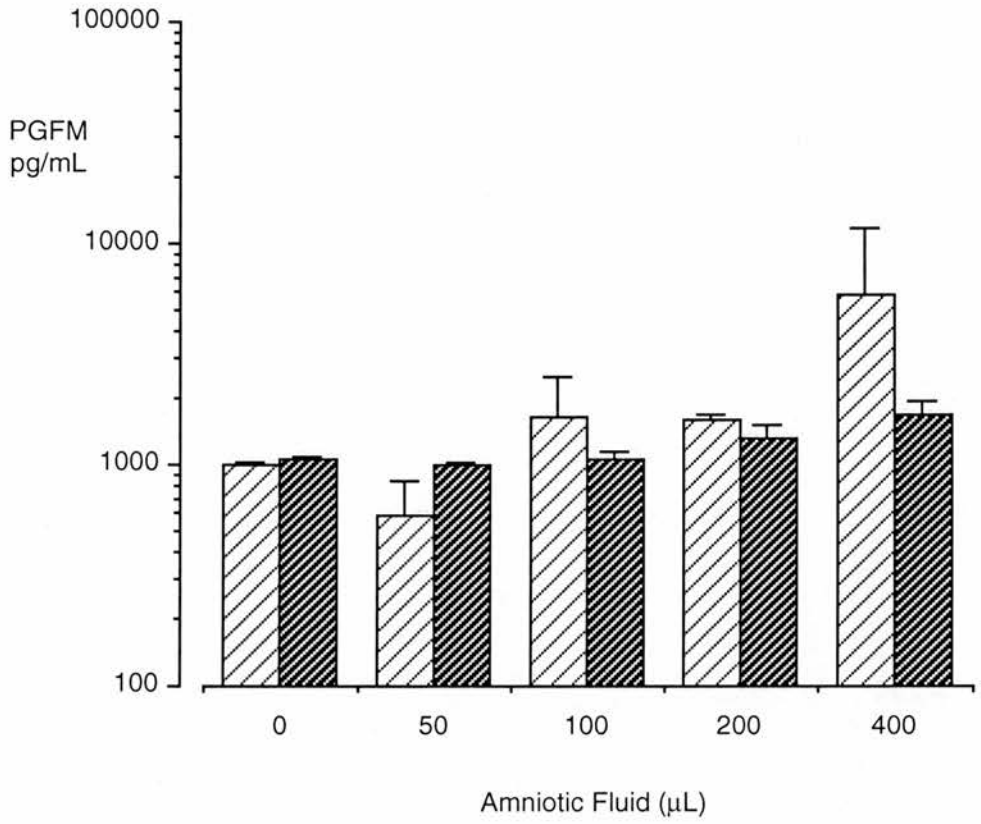
PGEM
pg/mL
1000
100
0
50
100
200
400
Amniotic Fluid (µL)

Figure 5.3


100000
PGFM
pg/mL
10000
1000
100
0
50
100
200
400
Amniotic Fluid (µL)

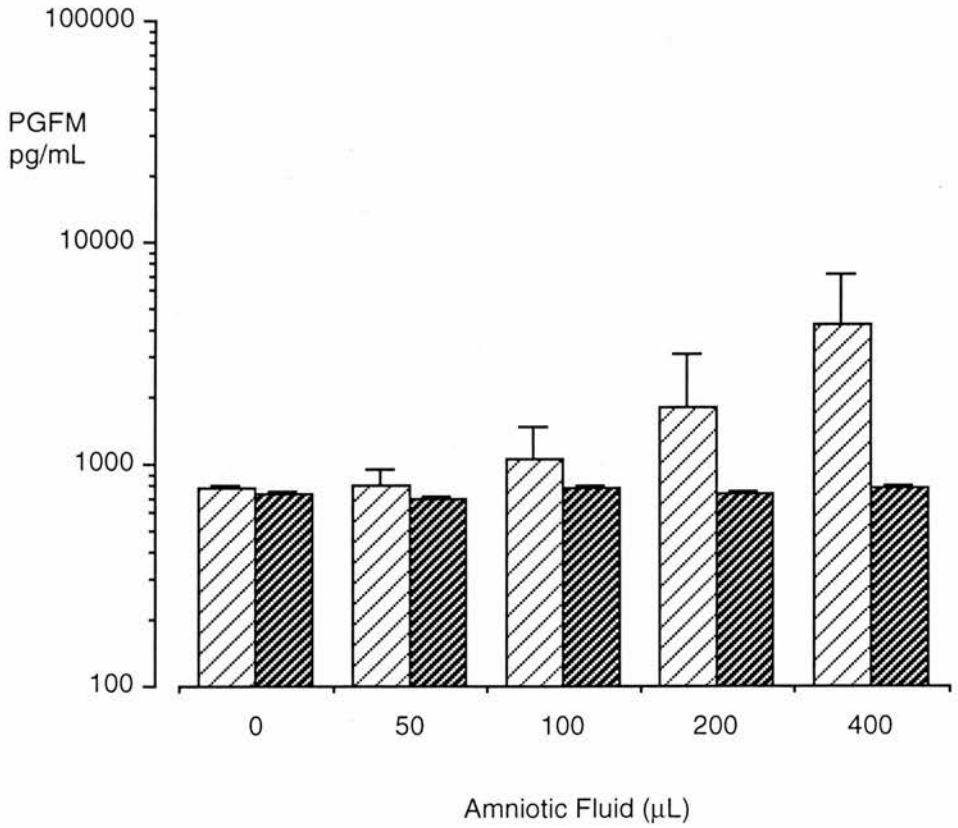

100000
PGFM
pg/mL
10000
1000
100
0
50
100
200
400
Amniotic Fluid (µL)

Figure 5.4

The effect of amniotic fluid on metabolism of exogenous prostaglandins by chorion cells:

Spontaneous labour and elective section amniotic fluid, in a dose of 400μL, significantly stimulated PGEM and PGFM production in chorion cell culture compared with control, $p<0.05$ (Fig. 5.5) The production of both PGEM and PGFM was significantly greater in the presence of spontaneous labour amniotic fluid compared with elective section fluid, $p<0.05$. In addition, the responsiveness of the chorion cells to phorbol myristoyl acetate, a stimulator of protein kinase C, is illustrated in Figure 5.5. In both cultures, amniotic fluid from elective section stimulated significantly more PGEM production compared with PGFM, $p< 0.0005$ (Fig. 5.6). Amniotic fluid from spontaneous labour stimulated significantly more PGFM production in comparison to PGEM, $p< 0.05$ (Fig. 5.6), but was not statistically significant in the second culture ($p= 0.056$). The ratio of PGFM to PGEM production was 0.38 for chorion cells cultured with elective section amniotic fluid, compared with and FM:EM ratio of 4.6 when cultured in the presence of spontaneous labour amniotic fluid. This represents a 12-fold increase in FM:EM ratio.

There was no difference in PGEM production by chorion, following the addition of 500ng PGE_2, when cultured in the presence of amniotic fluid from spontaneous labour or elective section compared with control (cells+500 ng PGE_2) in both cultures (Fig. 5.7). Similarly, there was no difference in the production of PGFM, following the addition of 500ng $PGF_{2\alpha}$, by chorion cells cultured in the presence of amniotic fluid from elective section (both cultures) (Fig. 5.8). Amniotic fluid from spontaneous labour had no effect on PGFM production, following addition of $PGF_{2\alpha}$, in one culture, and stimulated significantly greater PGFM production compared with control (cells+500 ng $PGF_{2\alpha}$) in the second culture, $p<0.05$.

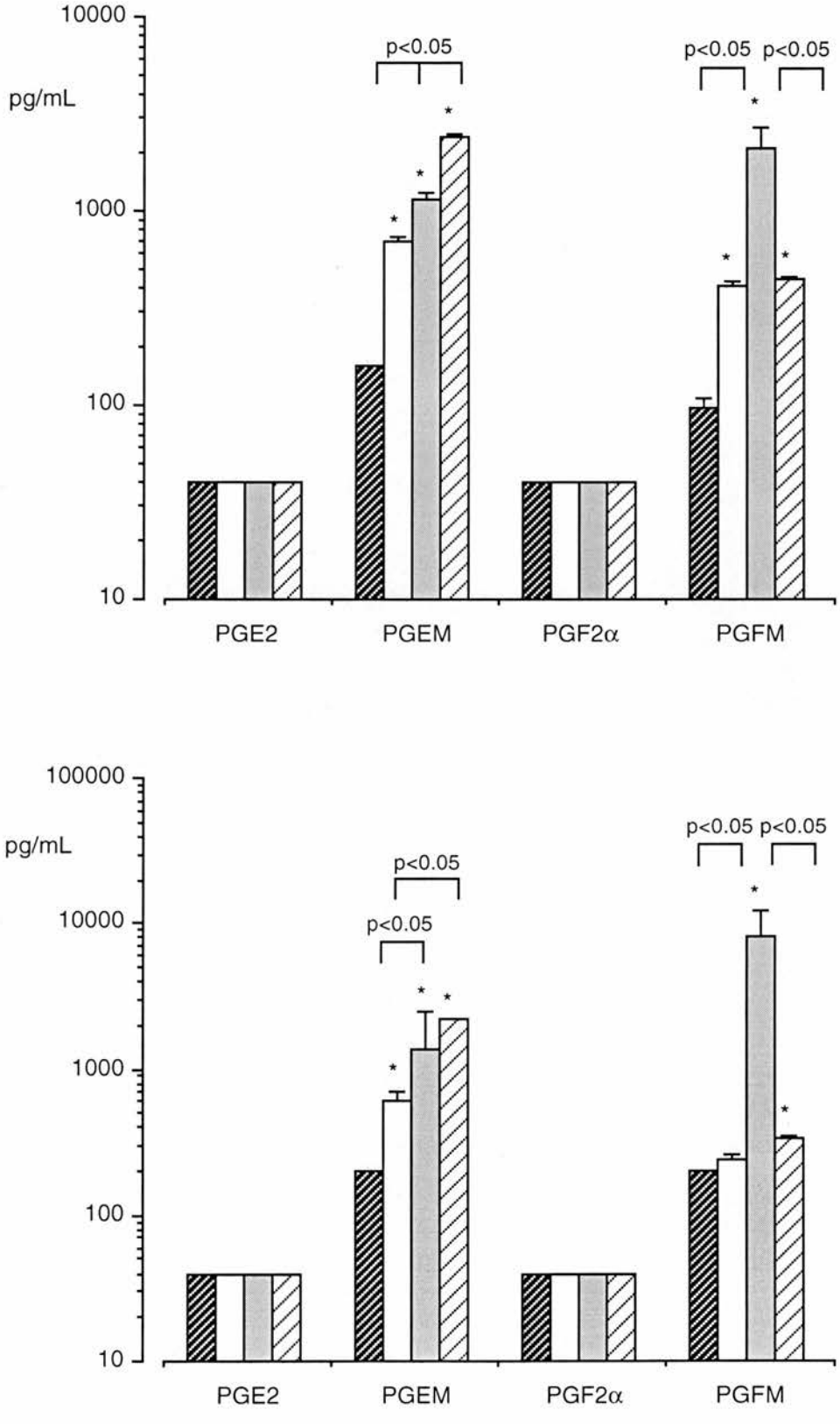
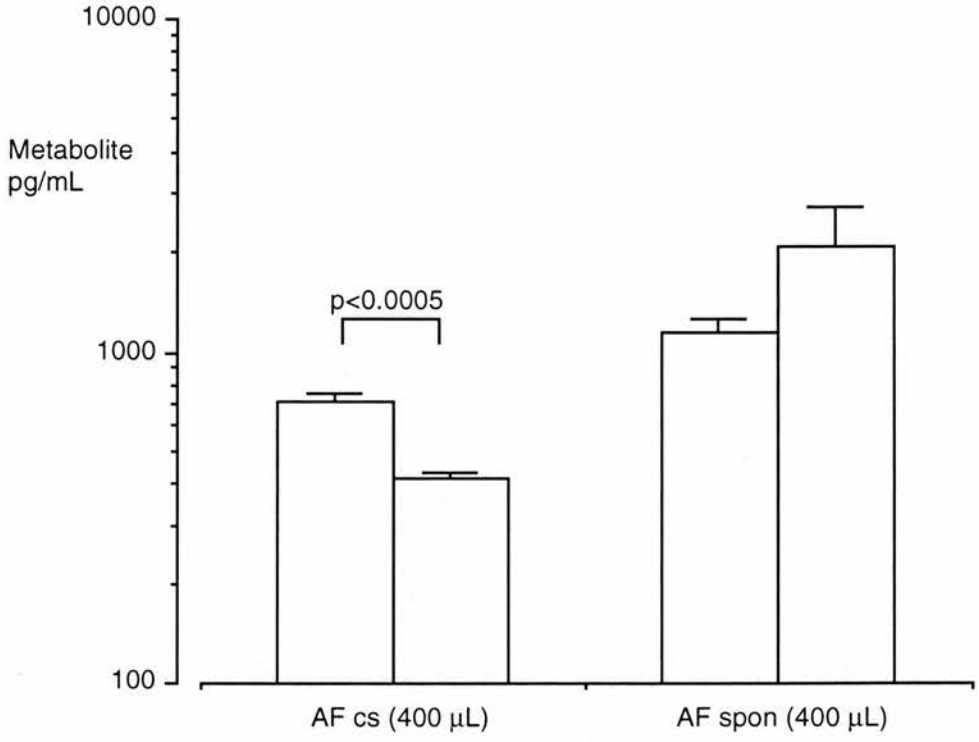
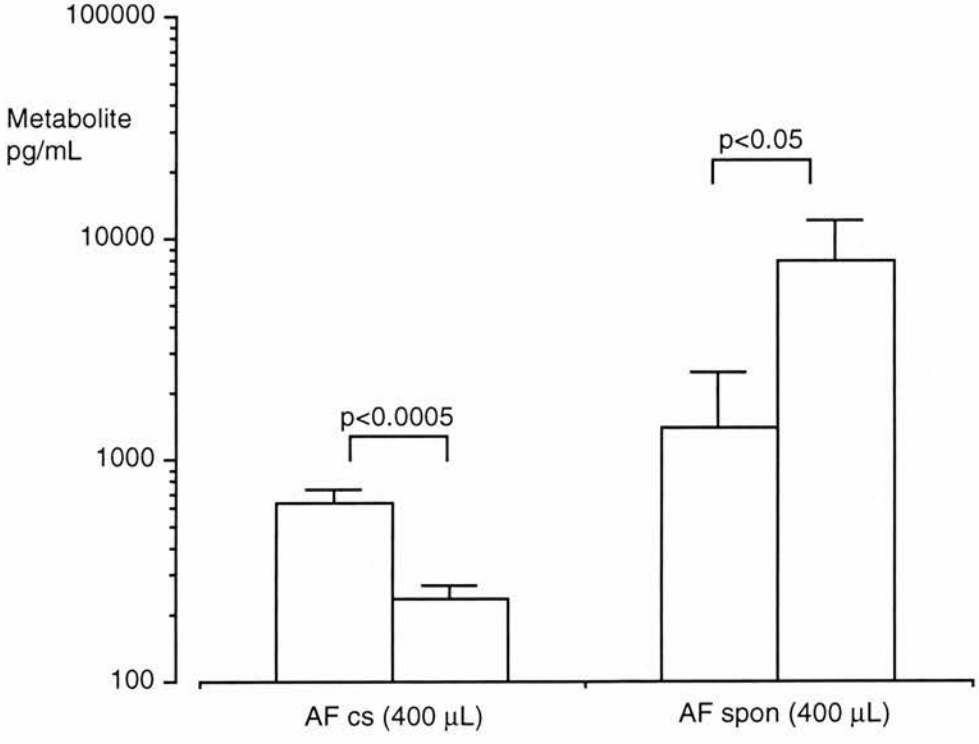

10000
pg/mL
1000
100
10
p<0.05
p<0.05
p<0.05
PGE2
PGEM
PGF2α
PGFM
100000
pg/mL
10000
1000
100
10
p<0.05
p<0.05
p<0.05
p<0.05
PGE2
PGEM
PGF2α
PGFM

Figure 5.5


10000
Metabolite
pg/mL
p<0.0005
1000
100
AF cs (400 µL)
AF spon (400 µL)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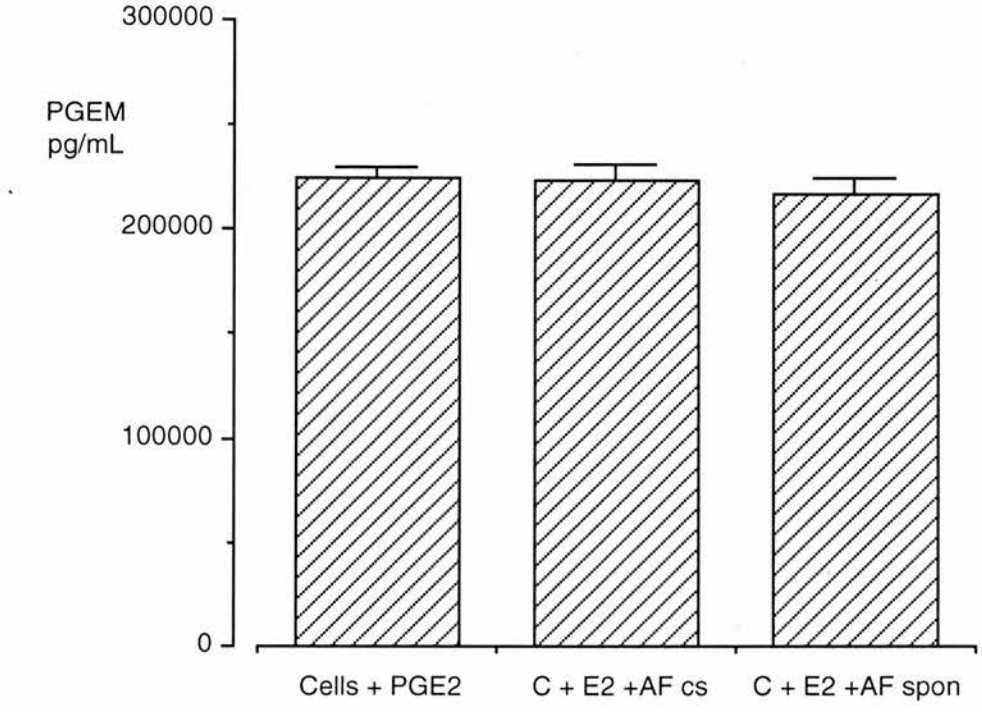
Metabolite
pg/mL
p<0.05
10000
p<0.0005
1000
100
AF cs (400 µL)
AF spon (400 µL)

Figure 5.6


300000
PGEM
pg/mL
200000
100000
0
Cells + PGE2
C + E2 +AF cs
C + E2 +AF spon

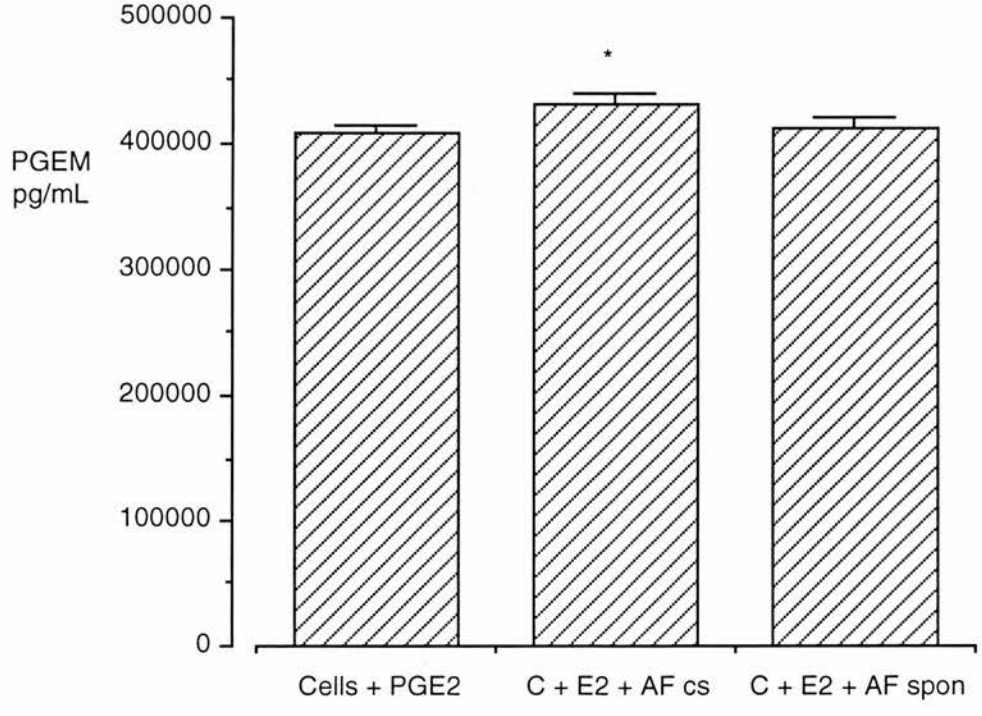
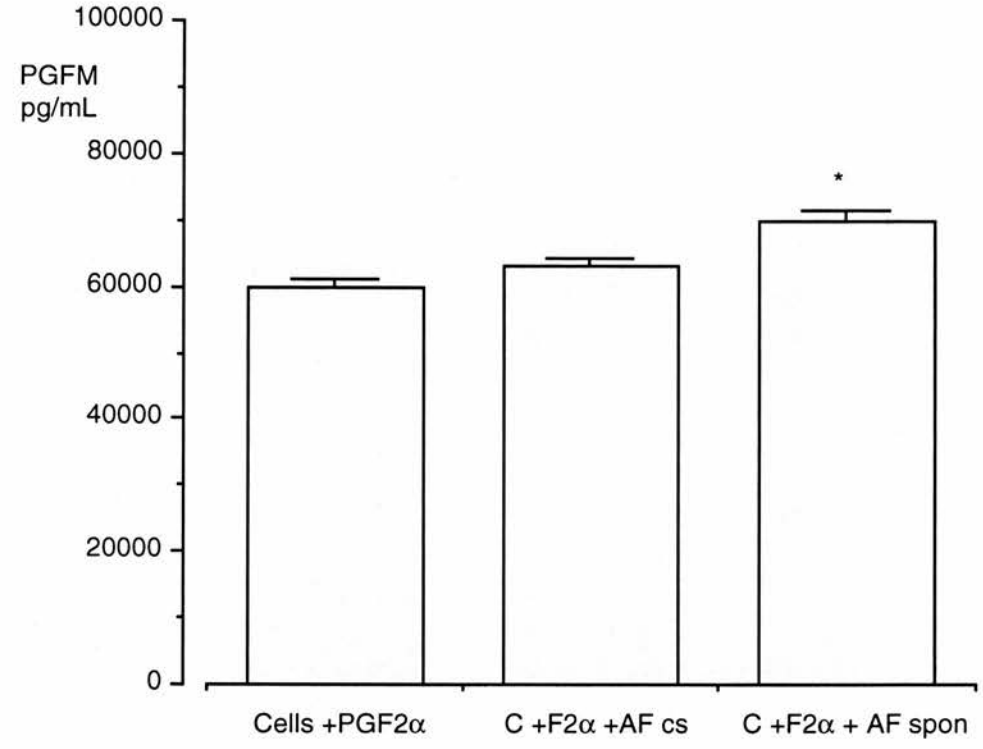

500000
*
PGEM
pg/mL
400000
300000
200000
100000
0
Cells + PGE2
C + E2 + AF cs
C + E2 + AF spon

Figure 5.7


100000
PGFM
pg/mL
80000
60000
40000
20000
0
*
Cells +PGF2α
C +F2α +AF cs
C +F2α + AF spon

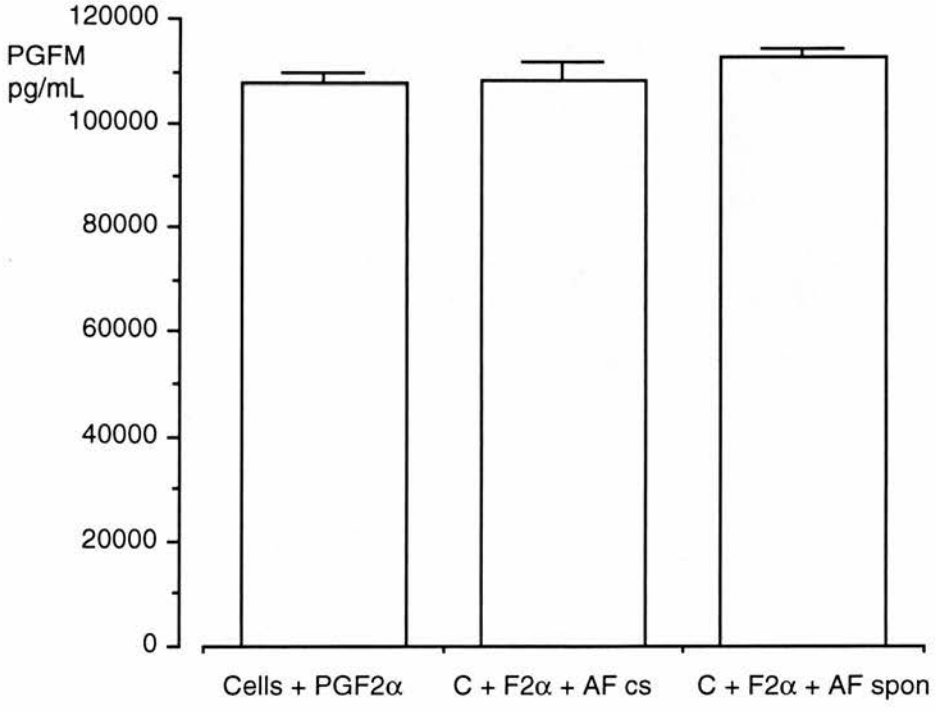

120000
PGFM
pg/mL
100000
80000
60000
40000
20000
0
Cells + PGF2α
C + F2α + AF cs
C + F2α + AF spon

Figure 5.8

Discussion

We have demonstrated that amniotic fluid from spontaneous labour and elective caesarean section at term is capable of stimulating prostaglandin production by amnion cell culture. This is in keeping with other workers who have shown increased PGE_2 production by amnion cells (Dowling et al, 1991) and bovine seminal vesicles (Cohen et al, 1985) incubated with amniotic fluid. In our experiments PGE_2 production was stimulated by amniotic fluid in a dose-dependent manner, confirming the findings of Mitchell et al (1984a) who demonstrated PGE_2 stimulatory activity in amniotic fluid at all gestations. However, these workers did not find a difference in PGE_2 production in relation to parturition, unlike our experiments where amniotic fluid obtained at spontaneous labour had a significantly greater stimulatory effect than elective section fluid. Similarly, $PGF_{2\alpha}$ production was stimulated in these experiments and again responded to amniotic fluid in a dose-dependent manner. The response to spontaneous labour fluid was significantly greater than elective section fluid. It has previously been demonstrated that amniotic fluid will stimulate $PGF_{2\alpha}$ production by amnion, chorion and decidua (Rehnstrøm et al, 1983) but in these experiments there was no difference between spontaneous labour and elective section fluid. Reddi et al (1987) revealed that amniotic fluid obtained late in labour significantly stimulated $PGF_{2\alpha}$ production by sheep seminal vesicle prostaglandin synthase, whereas fluid obtained before labour had no such effect. In our amnion cells caesarean section fluid had little effect on $PGF_{2\alpha}$ production, although a significant dose-response curve was achieved in one culture. In all cultures significantly more PGE_2 was produced compared with $PGF_{2\alpha}$ in response to both spontaneous labour and elective section fluid confirming that this is the major prostanoid synthesised by amnion (Okazaki et al, 1981a; Skinner and Challis, 1985).

There was some variability in the response of the chorion cell cultures to amniotic fluid. PGEM production, reflecting PGE_2 synthesis, was stimulated by spontaneous labour amniotic fluid in a dose-dependent manner in one culture. Elective section amniotic fluid had a dose-related stimulatory effect on PGEM production in both cultures. Similarly, PGFM production, reflecting $PGF_{2\alpha}$ synthesis, was stimulated by amniotic fluid from the two groups in both cultures. There was a significant difference in the stimulatory effect of spontaneous labour amniotic fluid compared with that of elective section with regard to PGFM production. In the metabolism studies we confirmed the stimulatory effect of amniotic fluid (400 μL) from spontaneous labour and elective section on PGEM and PGFM production in both chorion cultures, although elective section fluid had no significant effect on PGFM production in one culture. Spontaneous labour amniotic fluid stimulated significantly more PGEM and PGFM production compared with elective section fluid in both cultures. In all chorion cultures elective section fluid stimulated significantly greater PGEM production than PGFM, whereas spontaneous labour fluid, although not significant in all cultures, tended to favour PGFM production.

We have shown, therefore, that amniotic fluid obtained at elective section and spontaneous labour is capable of stimulating prostaglandin production by both amnion and chorion and that there is a preference for which prostaglandin is produced: spontaneous labour favours PGE_2 and PGFM production by amnion and chorion respectively, while elective section fluid stimulates PGE_2 synthesis by both tissues (reflected as PGEM in chorion). These findings are in keeping with the fact that under basal conditions PGEM is the main product of the chorion (Cheung and Challis, 1989), whereas $PGF_{2\alpha}$ is thought to be integral to the maintenance of established labour, with concentrations of this prostaglandin increasing as cervical dilatation advances (Keirse et al, 1974; MacDonald and Casey, 1993) There is a significant difference in the stimulatory activity of the amniotic fluid in relation to parturition, but

we do not know from these experiments whether this is a result of gestationally related changes in inhibitory or stimulatory properties of the fluid. These findings are not dissimilar from those of Reddi et al (1987) who found that as labour progressed there was a simultaneous reduction in stimulatory activity and increase in inhibitory activity in amniotic fluid with respect to PGE_2 production, whilst the converse was true for $PGF_{2\alpha}$ production.

It has been postulated that changes in the metabolic capacity of the chorion may be just as important in the regulation of prostaglandin concentrations as any direct stimulatory effect on prostaglandin synthesis (Khan et al, 1991). In this study amniotic fluid from spontaneous labour or elective section had no effect on the concentration of PGEM or PGFM recovered from the chorion cell cultures, following the addition of PGE_2 and $PGF_{2\alpha}$ respectively, compared with control (cells + PG alone). These findings suggest that amniotic fluid has no significant effect on the metabolic pathway for prostaglandins in chorion, and in particular that there is no reduction in metabolism in relation to labour which could result in the increased prostaglandin concentrations seen at this time. This is in keeping with previous reports showing no change in prostaglandin metabolism in association with spontaneous labour (Skinner and Challis, 1985) and the results discussed in chapter 4. This also accords with the finding that the localisation of immunoreactive PGDH in chorion does not change with labour (Cheung et al, 1990). In contrast, in cases of preterm labour associated with chorioamnionitis, it has been demonstrated that immunoreactive staining for PGDH is significantly reduced as a result of trophoblast destruction (Van Meir et al, 1996). This would enable prostaglandins to escape metabolism and thus facilitate myometrial contractility. In the present study, the metabolism by chorion of exogenous PGE_2 to its inactive metabolite PGEM was significantly greater than that of exogenous $PGF_{2\alpha}$ to PGFM ($p=0.0001$). The same was true in the presence of spontaneous labour amniotic fluid ($p<0.005$) and elective section fluid ($p=0.0001$). This is in keeping

with the fact that the prostaglandin metabolising enzymes are more able to inactivate PGE_2 compared with $PGF_{2\alpha}$ (Casey et al, 1989b; Ishihara et al, 1991).

We have confirmed that amniotic fluid can stimulate prostaglandin production by fetal membranes and demonstrated that it has no effect on prostaglandin metabolism in these tissues. What factor(s) present in amniotic fluid is responsible for the increased production has still to be clarified. It has been hypothesised that, since amniotic fluid at term is largely composed of fetal urine, the signal for the onset of parturition may come from the appropriately mature fetus. (Mitchell, 1984b) Fetal urine has been shown to stimulate PGE_2 production by amnion in a concentration-dependent manner (Casey et al, 1983), and urine obtained from fetuses following spontaneous labour stimulates greater production of PGE_2 compared with fetuses delivered by elective caesarean section (Strickland et al, 1982). Alternatively, recruitment of mediators such as interleukins and TNFα, resulting in an inflammatory process, may play a role in the onset of parturition, and prostaglandins will enhance the inflammatory role of such cytokines. Indeed, such a mechanism has been proposed for the changes associated with cervical ripening (Liggins, 1981). An example of cytokine recruitment is the increased concentration of IL-1β in amniotic fluid which has been reported in women in preterm labour associated with infection (Romero et al, 1989e) and in women in spontaneous labour at term (Romero et al, 1990a), and this cytokine is known to be capable of stimulating PGE_2 production by amnion cell culture (Mitchell et al, 1993b).

Although we have looked at prostaglandin synthesis and metabolism by fetal membranes in parallel, it must be remembered that the *in vitro* situation is not necessarily a true reflection of events occurring *in vivo*. The amnion and chorion were separated in these experiments and therefore any modulatory effect that these tissues may exert on each other will have been lost. Chorion-conditioned medium stimulates PGE_2 production by amnion (Lundin-Schiller et al, 1990b), and chorion cells

themselves are capable of interleukin production (Lundin-Schiller and Mitchell, 1991b). Importantly, the membranes were devoid of decidua, which may influence these avascular tissues in a paracrine fashion. In particular, studies have shown that a decidual product is capable of inhibiting prostaglandin production by amnion (Romero et al, 1987) and that decidual cells contain an inhibitor of cyclooxygenase I (Sun et al, 1994).

In summary, these experiments confirm the stimulatory effect of amniotic fluid on prostaglandin production by amnion and chorion and demonstrate that this activity is greater in association with spontaneous labour. However, labour had no effect on the metabolising capacity of the chorion suggesting that it is increased production rather than a reduction in prostaglandin metabolism which is responsible for the increased prostaglandin concentrations related to parturition.

Chapter Six

Mechanisms Involved in the Stimulatory Effect of Amniotic Fluid on Prostaglandin Production by Human Fetal Membranes.

Introduction.

The amnion is the most extensively investigated of the intrauterine tissues with regard to the control of prostaglandin production. Since prostaglandins are not stored, but rather released immediately following synthesis in response to external stimuli, the major control of prostaglandin action lies with the regulation of its synthesis. The release of arachidonic acid, the obligatory precursor of prostaglandins of the 2-series, from membrane phospholipids is thought to be a rate-limiting step in prostaglandin synthesis. Protein kinase C (PKC) is a calcium- and lipid-dependent enzyme which has a crucial role in cell surface signal transduction, leading to the activation of many cellular functions. PKC activity has been identified in human amnion, and it has been suggested that PKC participates in the mobilisation of arachidonic acid, and the activation of cyclooxygenase (COX), thus facilitating prostaglandin synthesis.

Two isoenzymes of COX have been identified and each enzyme is the product of a separate gene (Xie et al, 1993; Wen et al, 1993). COX-1 is constitutively expressed in fetal membranes, whereas COX-2 is the inducible form of the enzyme whose expression can be stimulated by a number of agonists (O'Sullivan et al, 1992; Mitchell et al, 1993b). COX activity is known to increase with advancing gestation, with a further increase in association with labour (Teixeira et al, 1993). Investigation of mRNA levels for COX-1 and 2 in human amnion has revealed that COX-2 mRNA, but not COX-1 mRNA, is increased in association with labour onset, suggesting that increased COX-2 expression is responsible for the increased COX activity seen at this time (Hirst et al, 1995). This finding of increased COX-2 expression in association with labour has been confirmed by other investigators (Slater et al, 1995), lending further support to the theory that COX-2 activity may be of principle importance in producing the increase in prostaglandin concentrations seen with the onset of labour.

The purpose of this study is to investigate potential mechanisms involved in the stimulatory effect of amniotic fluid on prostaglandin production by fetal membranes.

Subjects and Methods.

Amniotic fluid was collected from two groups of pregnant women at term (37-42 weeks) with an uncomplicated pregnancy. The first group laboured spontaneously and achieved a vaginal delivery without oxytocin augmentation (n=6); the second group was delivered by elective caesarean section for either breech presentation or previous caesarean section (n=6). Fetal membranes were collected from a third group of women undergoing uncomplicated elective caesarean section at term (n=2).

Mechanisms of stimulation of prostaglandin production by amniotic fluid:

Fetal membranes were collected from women undergoing elective caesarean section and amnion and chorion cell cultures prepared as described in section 2.2.1. A cell density of $2x10^5$ per well was employed The plates were incubated in humidified 95% air; 5% CO_2 at 37 C for 24 hours. Amniotic fluid obtained at elective caesarean section (n=3) and following spontaneous labour and delivery (n=3) was used in the experiment. 400 μL of amniotic fluid was added to each well before addition of the following treatments (final concentrations): 1 μM and 10 μM staurosporine (inhibitor of PKC); 1 μM and 10 μM genestein (inhibitor of tyrosine kinase); 2 μg and 4 μg actinomycin D (inhibitor of transcription); 10 μg and 20 μg cycloheximide (inhibitor of translation). The doses of the above agents were chosen because they are within the range expected to inhibit prostaglandin production should the stimulatory effect of amniotic fluid be dependent on transcription, translation, protein kinase C or tyrosine

kinase alone or in combination (Zakar and Olson, 1988; Zakar and Olson, 1992; Pollard et al, 1993). The effect of a 10-fold increase in dose was studied for all agents apart from actinomycin D owing to limited substrate and the fact that the effect of actinomycin D on radiolabelled amino acid uptake plateaus at concentrations 4 μg/mL (Zakar and Olson, 1988).

Cells were incubated in culture medium alone and in the presence of amniotic fluid to act as control. In addition, 400 μL of amniotic fluid was incubated alone for estimation of background primary prostaglandin and metabolite concentrations. The final incubation volume in all wells was 1 mL and all experiments were performed in duplicate. The plates were incubated for a further 24 hours in humidified 95% air; 5% CO_2 at 37 C. The incubation medium was oximated and stored pending radioimmunoassay. The experiment was repeated on a second set of amnion and chorion cell cultures with different amniotic fluids (again, n=3 for each group).

Radioimmunoassay.

The competitive binding radioimmunoassay described in section 2.6 was employed in these experiments.

Statistical Analysis

One and two factor analysis of variance (ANOVA) were used to analyse the data. Where the data were not normally distributed, log transformation was employed prior to ANOVA. Background prostaglandin and metabolite concentrations in amniotic fluid were subtracted in all experiments.

Results.

Control (Fig. 6.1)

Spontaneous labour and elective section amniotic fluid significantly stimulated PGE_2 production by amnion in both cultures ($p<0.05$), and this effect was significantly greater in the presence of spontaneous labour fluid compared with elective section fluid ($p<0.05$). The same was true for PGEM production by chorion cells in both cultures ($p<0.05$). Spontaneous labour amniotic fluid significantly stimulated PGFM production by chorion in both cultures ($p<0.05$), but elective section amniotic fluid had no effect in either culture.

Cycloheximide (Figs. 6.2, 6.3)

Cycloheximide significantly inhibited baseline PGE_2 production by amnion ($p<0.05$). The stimulatory effect of amniotic fluid was also significantly inhibited but not abolished in the presence of cycloheximide, at both concentrations, compared with control ($p<0.05$).

Similarly, cycloheximide, 10 and 20 μg, significantly inhibited baseline PGEM production by chorion, and significantly inhibited PGEM production previously stimulated by spontaneous labour and elective section amniotic fluid ($p<0.05$). Cycloheximide, at either concentration, had no effect on baseline or amniotic fluid-stimulated PGFM production by chorion.

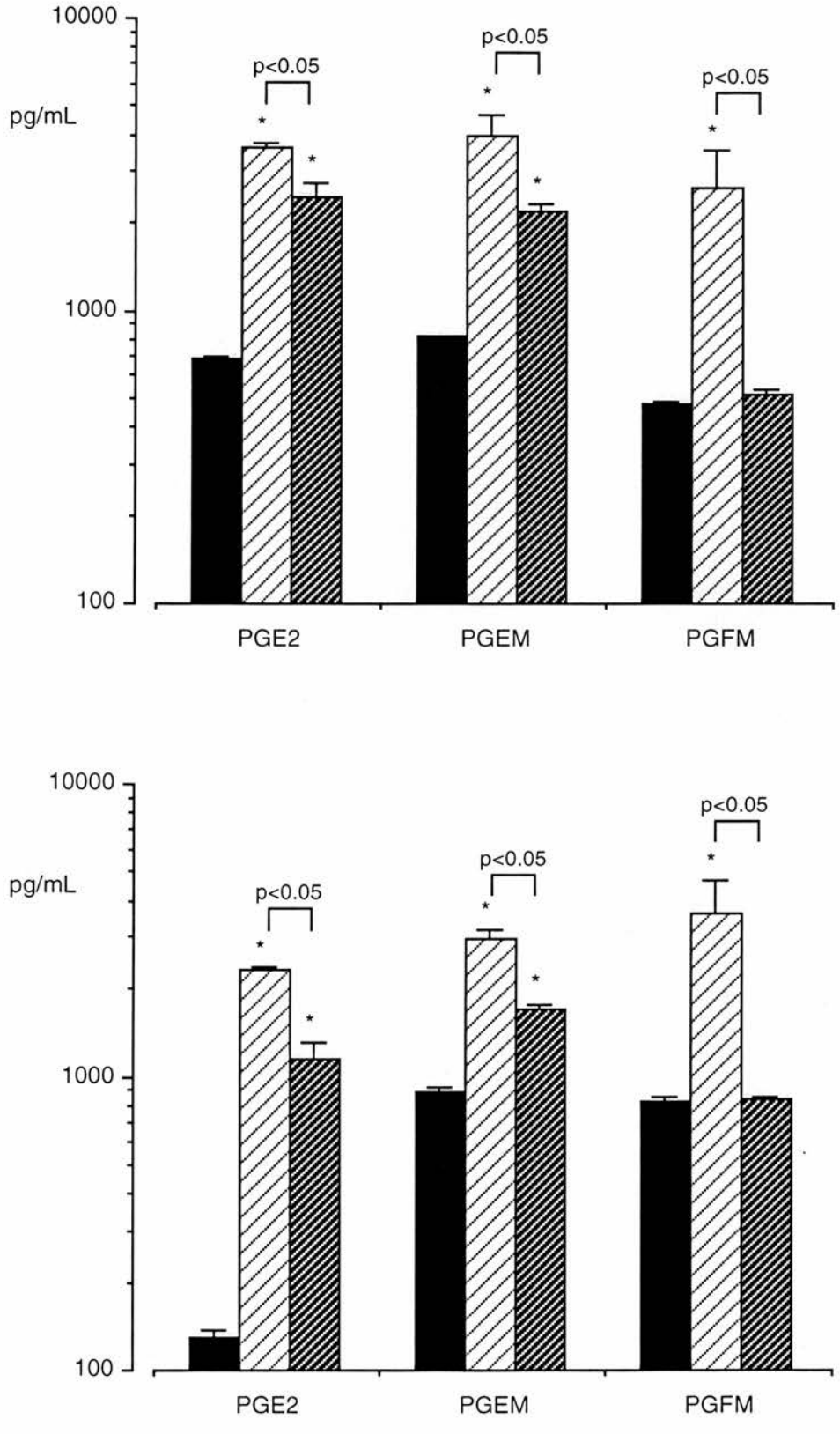
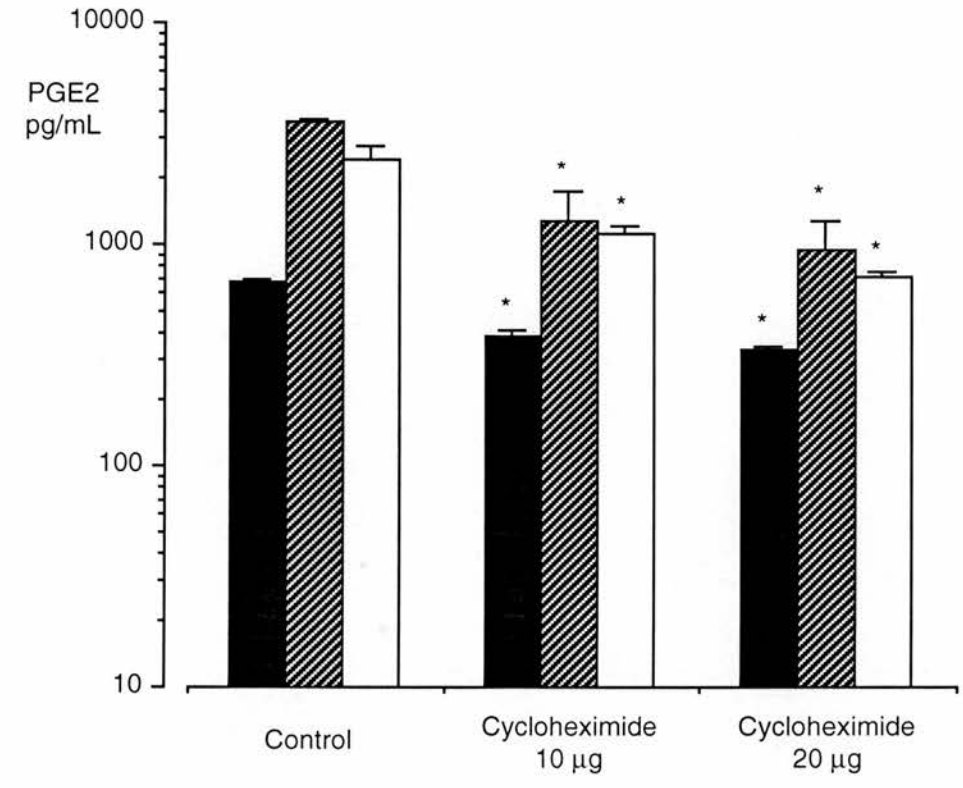
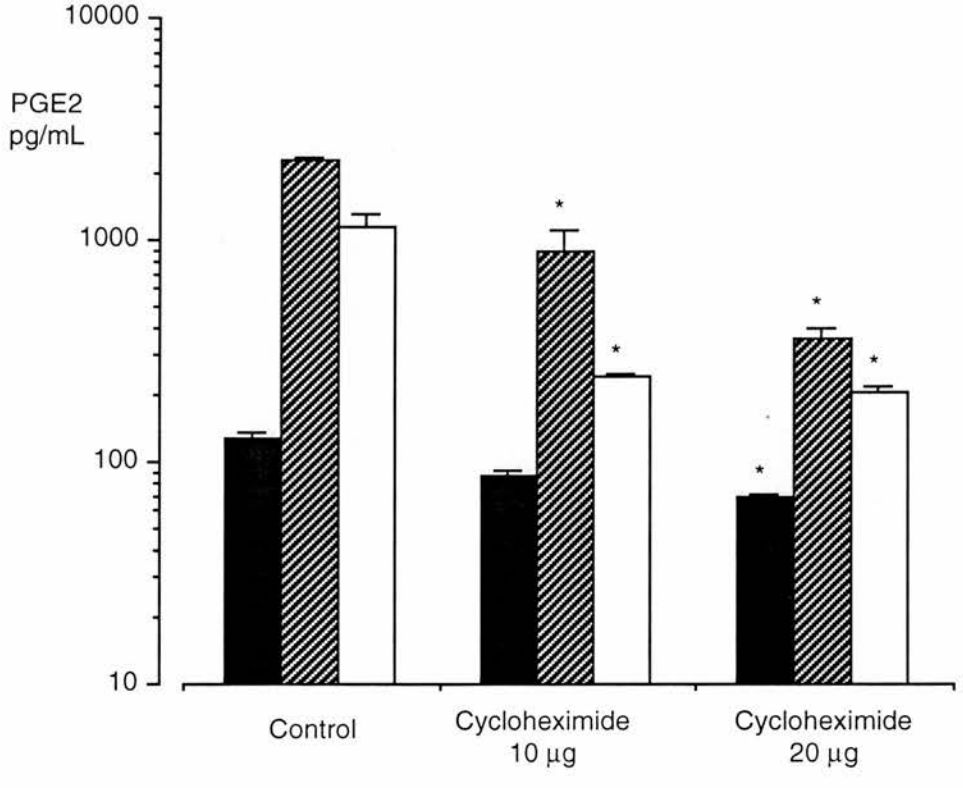

10000
pg/mL
1000
100
p<0.05
p<0.05
p<0.05
*
PGE2
PGEM
PGFM
10000
pg/mL
1000
100
p<0.05
p<0.05
p<0.05
PGE2
PGEM
PGFM

Figure 6.1


10000
PGE2
pg/mL
1000
100
10
Control
Cycloheximide
10 µg
Cycloheximide
20 µg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PGE2
pg/mL
1000
100
10
Control
Cycloheximide
10 µg
Cycloheximide
20 µg

Figure 6.2

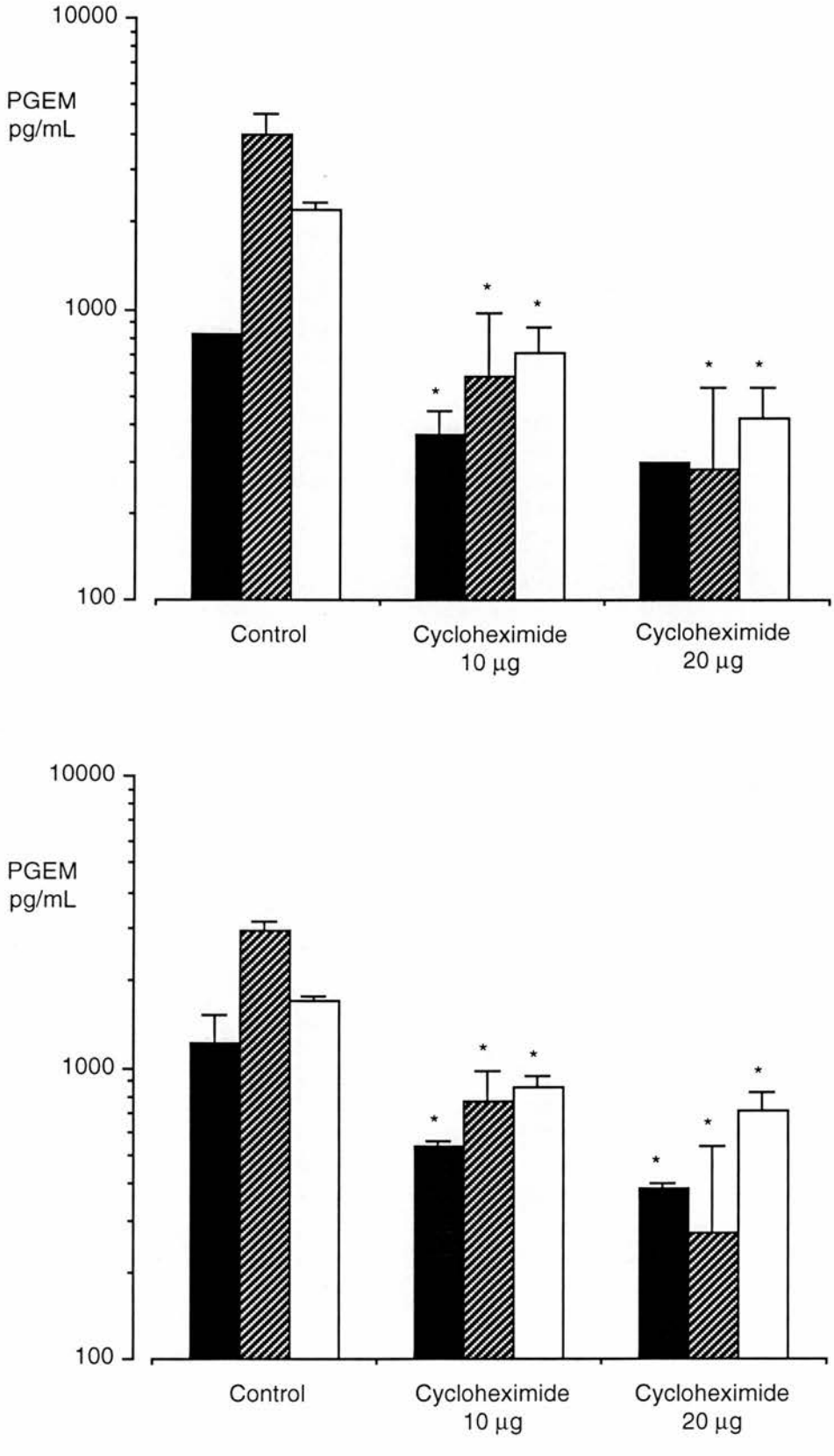

10000
PGEM
pg/mL
1000
100
Control
Cycloheximide
10 µg
Cycloheximide
20 µg
10000
PGEM
pg/mL
1000
100
Control
Cycloheximide
10 µg
Cycloheximide
20 µg

Figure 6.3

Actinomycin D (Figs. 6.4, 6.5)

Actinomycin D, 2 and 4 μg, significantly inhibited baseline PGE_2 production by amnion ($p<0.05$). The stimulatory effect of spontaneous labour amniotic fluid was significantly inhibited at doses of 2 and 4 μg, and that of elective section fluid was inhibited at a dose of 4 μg ($p<0.05$).

Actinomycin D had no effect on baseline PGEM production by chorion, but significantly inhibited the stimulatory effect of spontaneous labour amniotic fluid at doses of 2 and 4 μg ($p<0.05$). However, there was a variabiltiy in the response between different cell cultures in that actinomycin D did not inhibit the stimulatory effect of spontaneous labour amniotic fluid in the second cell culture. Actinomycin D 2 μg inhibited baseline PGFM production ($p<0.05$), but otherwise had no effect on PGFM.

Genestein (Figs. 6.6, 6.7)

Genestein 10 μM significantly inhibited baseline PGE_2 production ($p<0.05$). In addition, the stimulatory effect of amniotic fluid from both groups was significantly inhibited but not abolished by genestein 10 μM.

Genestein inhibited baseline PGEM production ($p<0.05$) and the stimulatory effect of spontaneous labour amniotic fluid was inhibited by genestein 1 and 10 μM ($p<0.05$). Genestein had no effect on elective section amniotic fluid-stimulated PGEM production. Genestein had no effect on PGFM production by chorion.

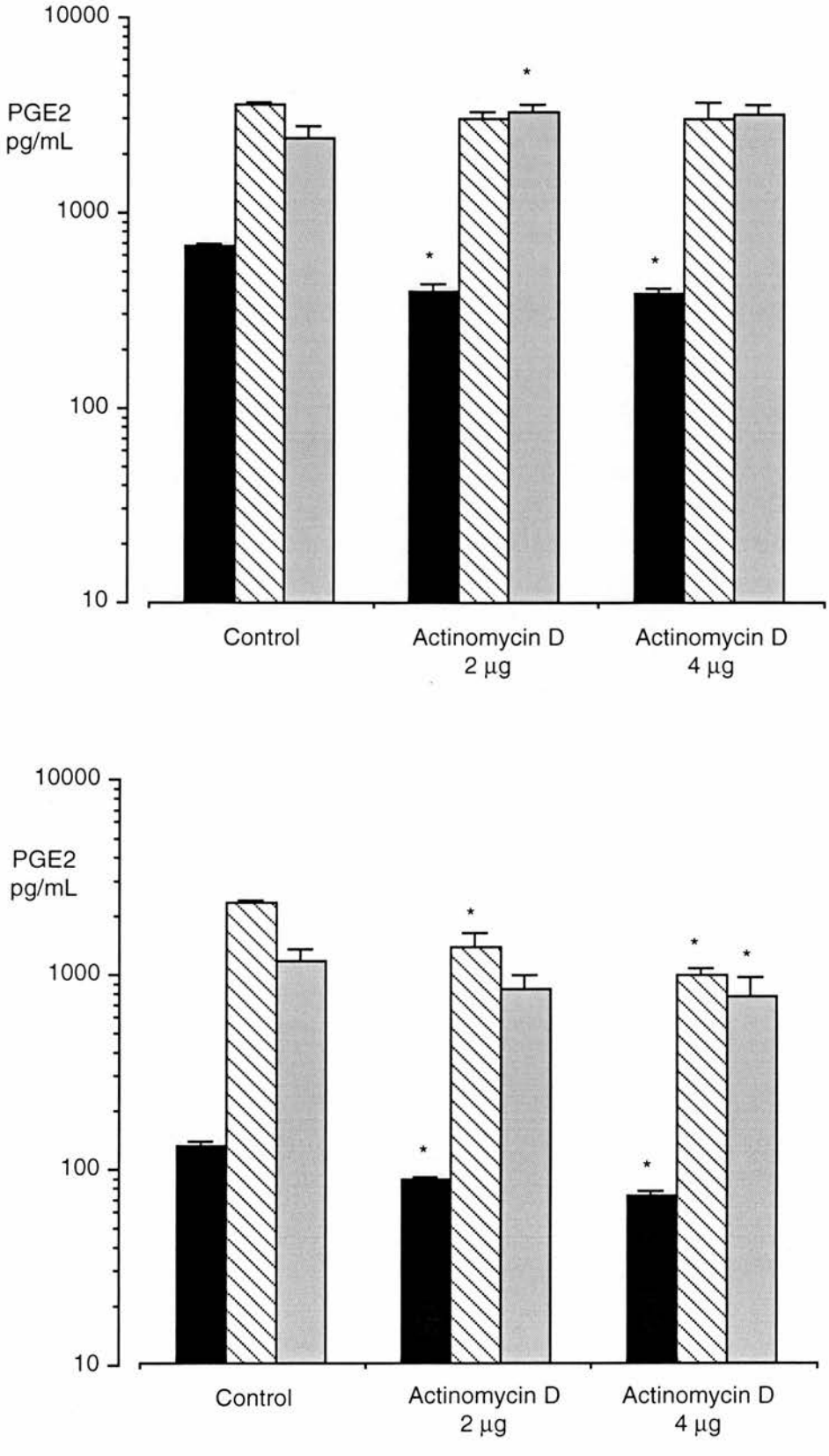

10000
PGE2
pg/mL
1000
100
10
*
*
*
Control
Actinomycin D
2 µg
Actinomycin D
4 µg
10000
PGE2
pg/mL
1000
100
10
*
*
*
*
Control
Actinomycin D
2 µg
Actinomycin D
4 µg

Figure 6.4

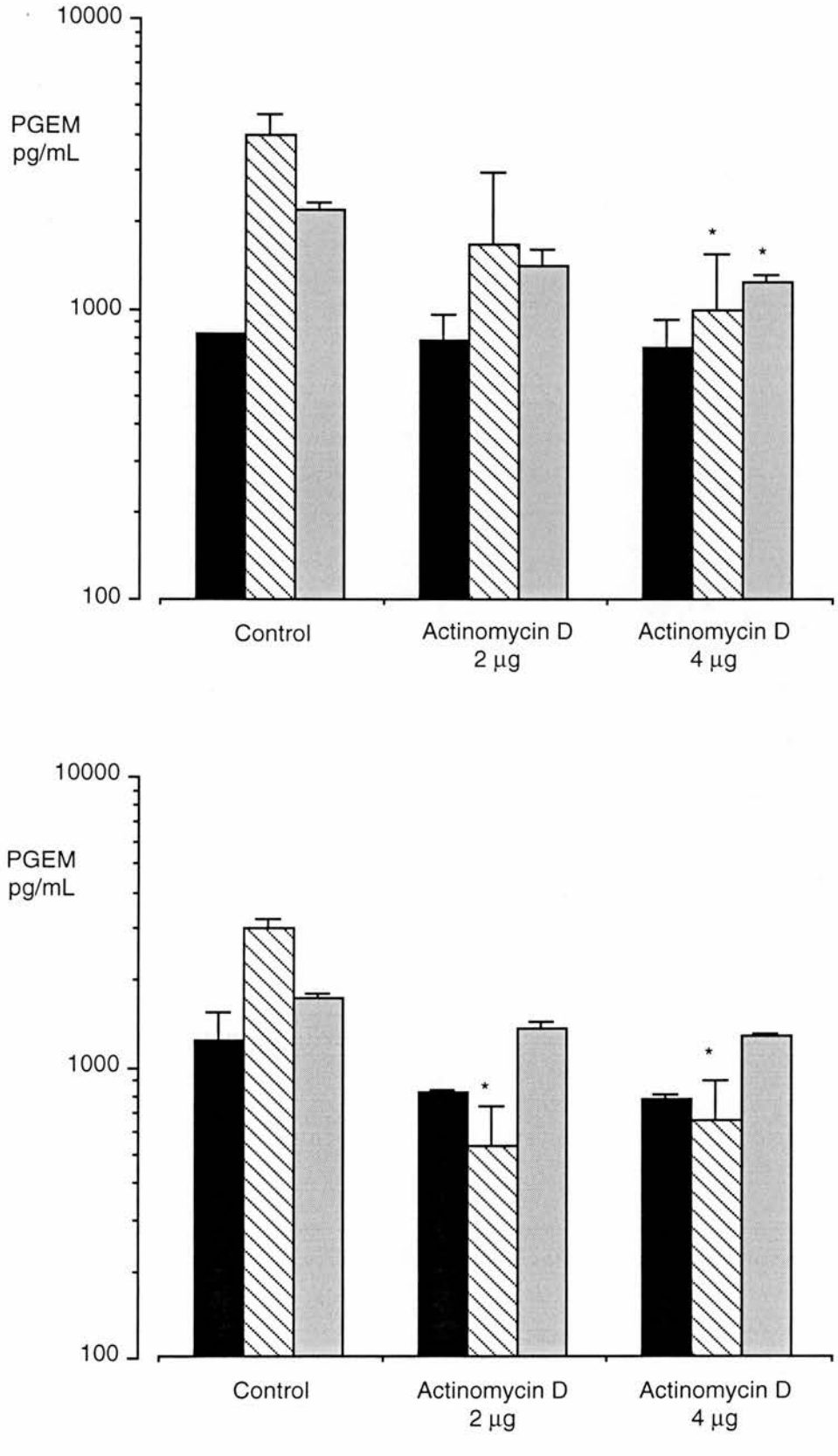

10000
PGEM
pg/mL
1000
100
Control
Actinomycin D
2 µg
Actinomycin D
4 µg
*
*
10000
PGEM
pg/mL
1000
100
Control
Actinomycin D
2 µg
Actinomycin D
4 µg
*
*

Figure 6.5

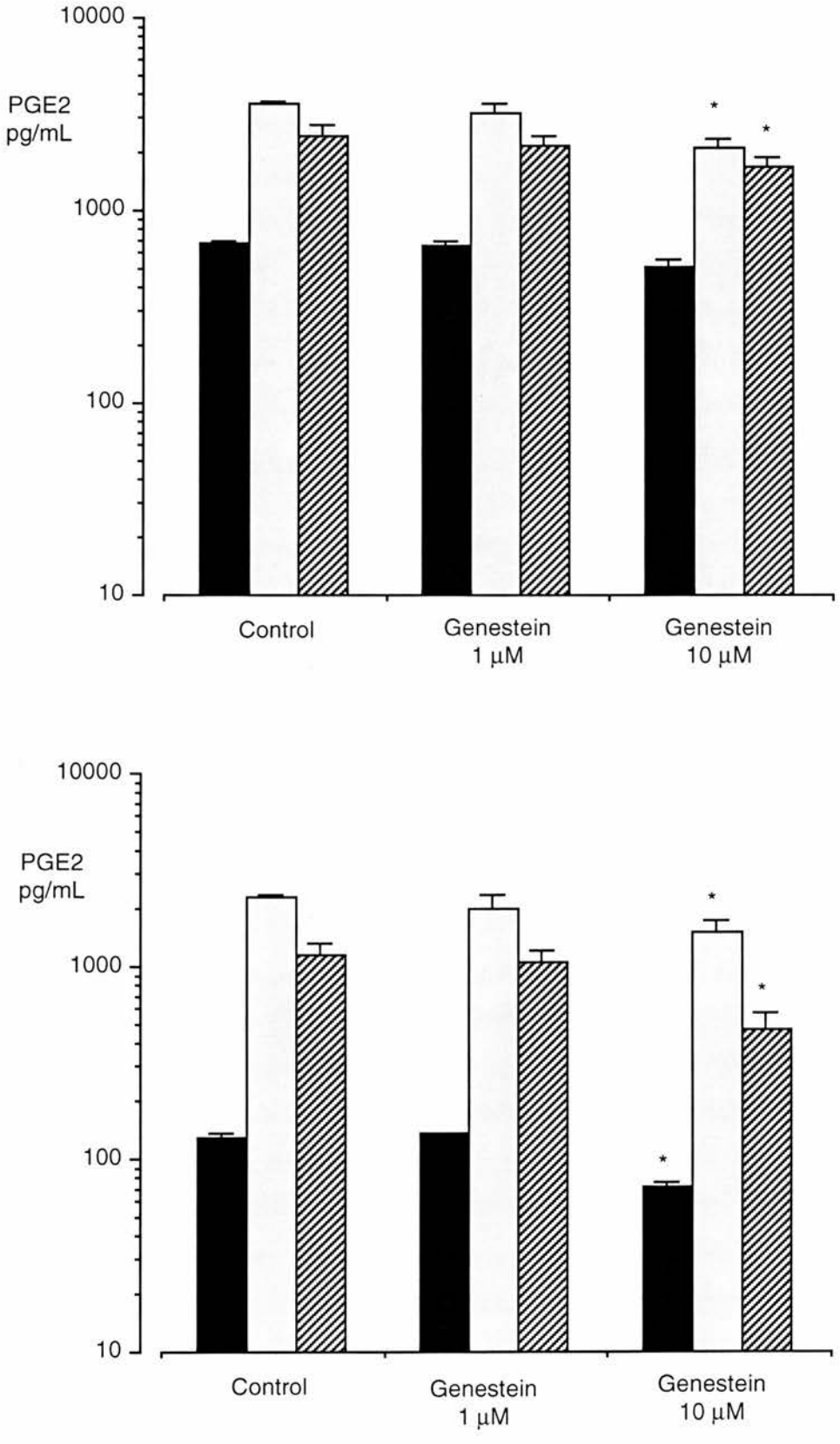
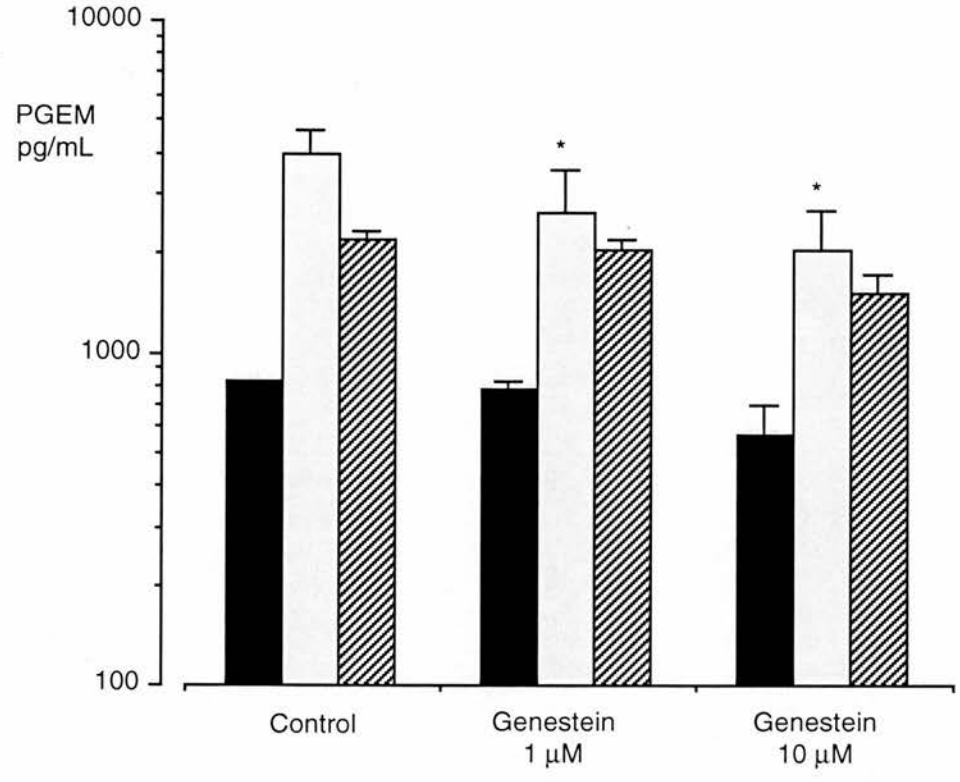

10000
PGE2
pg/mL
1000
100
10
*
*
Control
Genestein
1 μM
Genestein
10 μM
10000
PGE2
pg/mL
1000
100
10
*
*
*
Control
Genestein
1 μM
Genestein
10 μM

Figure 6.6


10000
PGEM
pg/mL
1000
100
*
*
Control
Genestein
1 μM
Genestein
10 μM

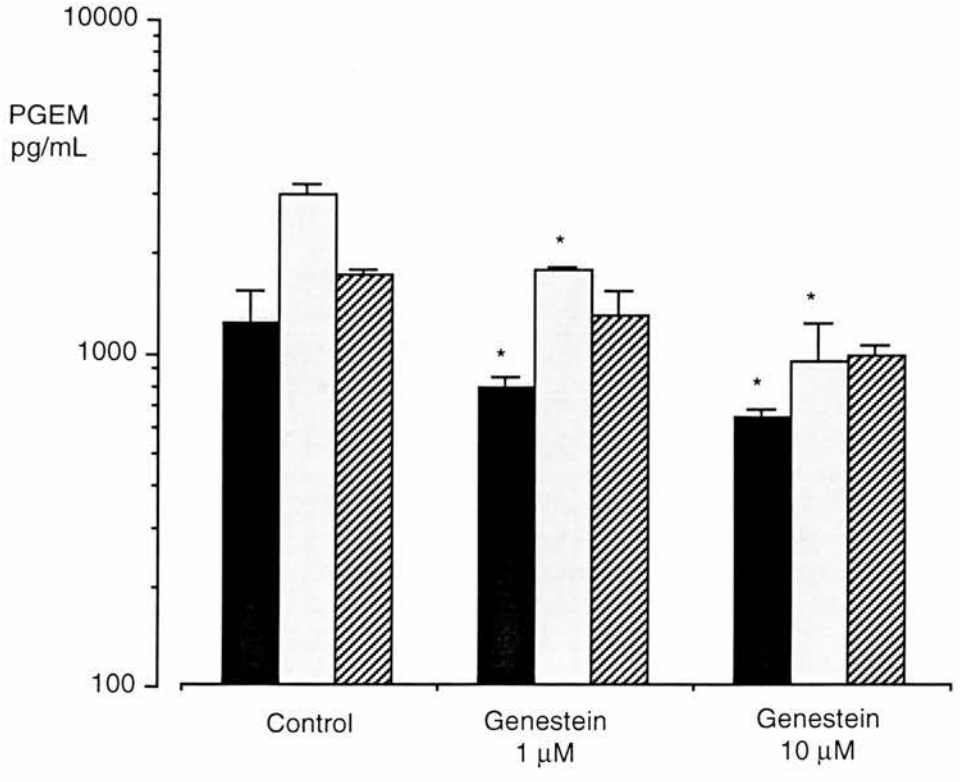

10000
PGEM
pg/mL
1000
100
*
*
*
*
Control
Genestein
1 μM
Genestein
10 μM

Figure 6.7

Staurosporine (Figs. 6.8, 6.9)

Staurosporine, 1 and 10 μM, significantly inhibited baseline PGE_2 production ($p<0.05$). The stimulatory effect of amniotic fluid from both groups was also inhibited in the presence of staurosporine ($p<0.05$). At a dose of 10 μM staurosporine completely abolished the stimulatory effect of elective section amniotic fluid. This was not the case for spontaneous labour fluid which continued to stimulate greater PGE_2 production compared with cells treated with staurosporine alone.

Staurosporine, 1 and 10 μM, significantly inhibited baseline PGEM production by chorion ($p<0.05$), and also inhibited the stimulatory effect of amniotic fluid from both spontaneous labour and elective section ($p<0.05$). Staurosporine had no effect on baseline PGFM production by chorion, but significantly inhibited the stimulatory effect of spontaneous labour amniotic fluid ($p<0.05$).

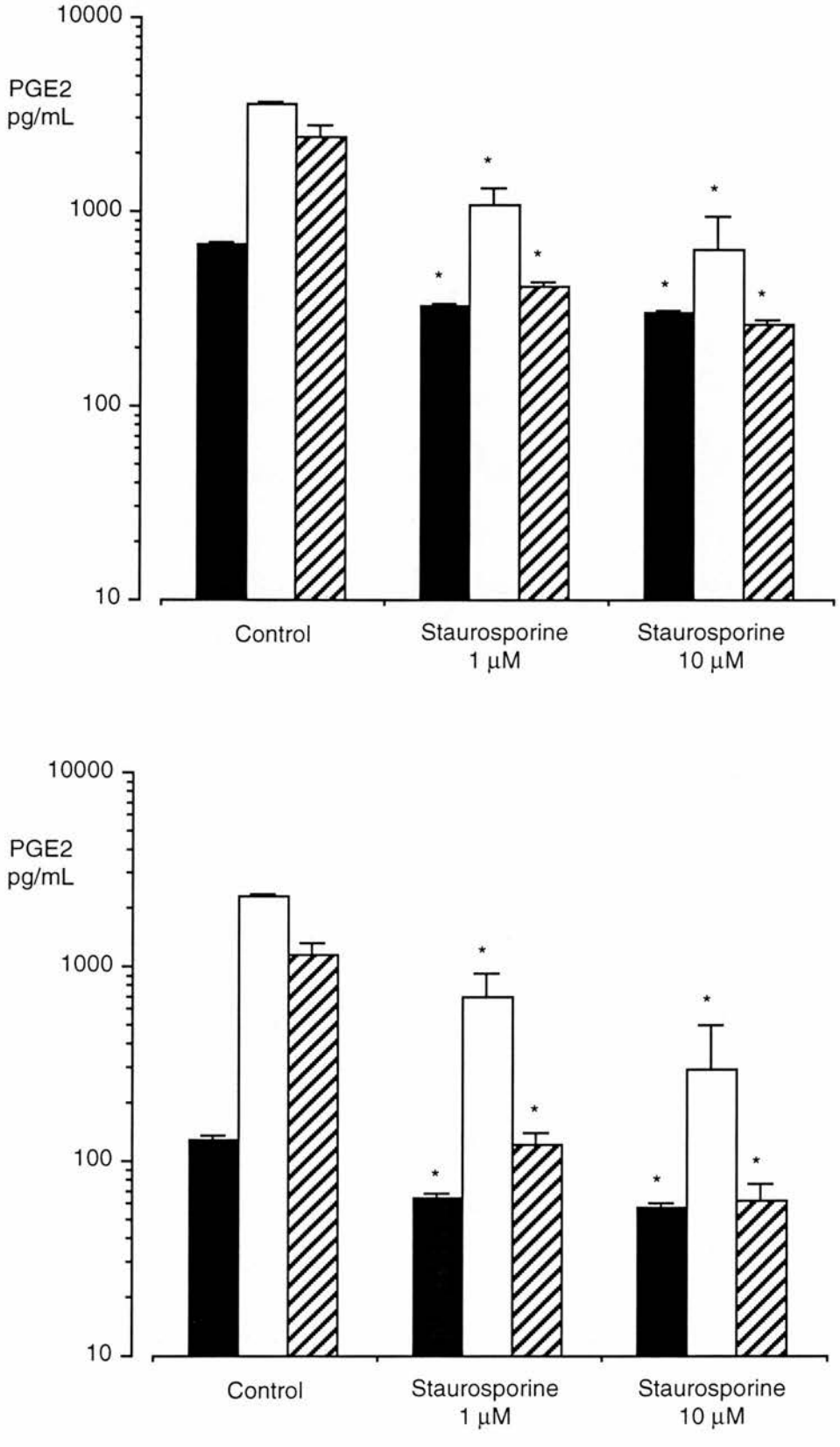

10000
PGE2
pg/mL
1000
100
10
Control
Staurosporine
1 μM
Staurosporine
10 μM
10000
PGE2
pg/mL
1000
100
10
Control
Staurosporine
1 μM
Staurosporine
10 μM

Figure 6.8

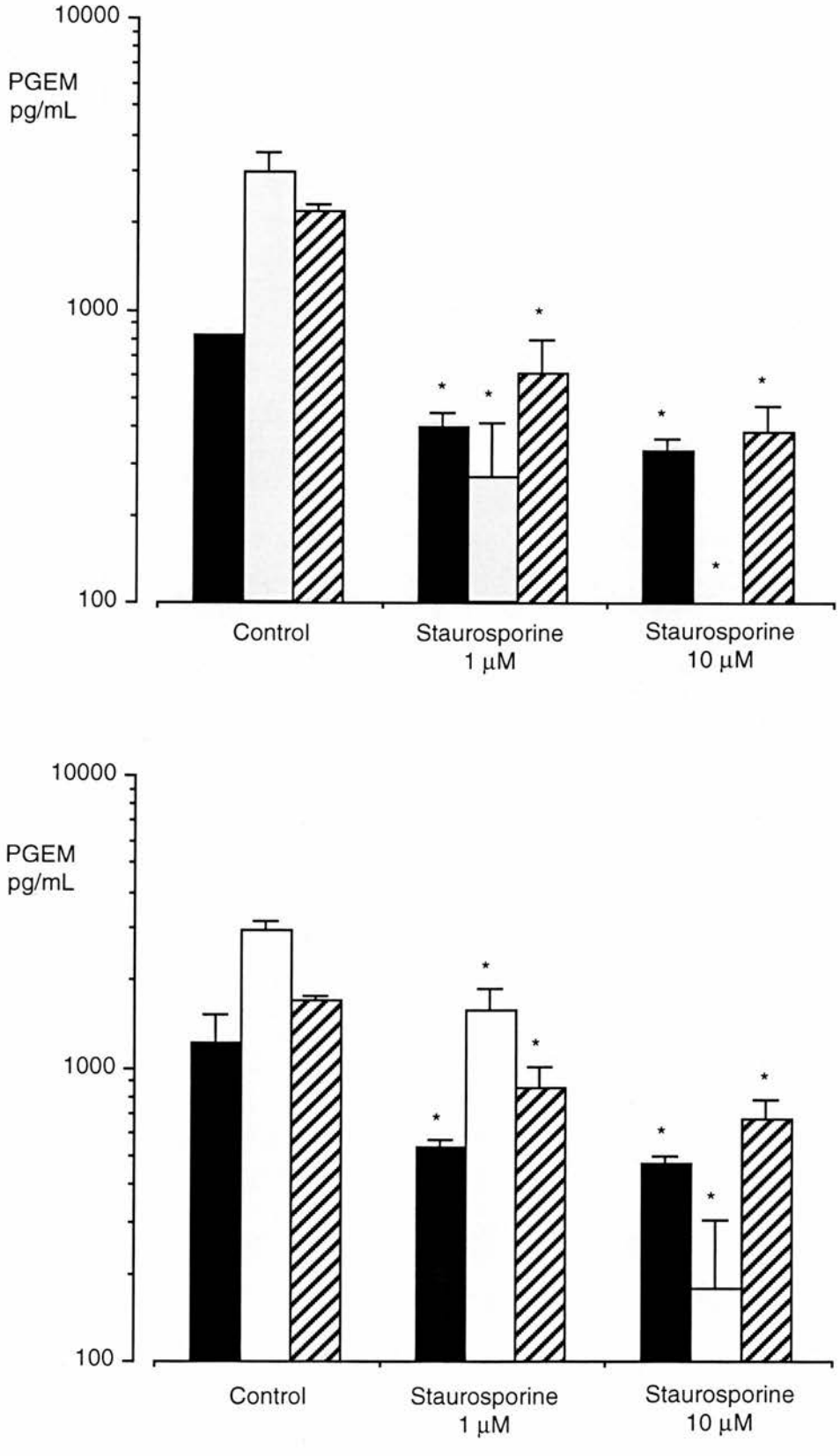

10000
PGEM
pg/mL
1000
100
Control
Staurosporine
1 μM
Staurosporine
10 μM
10000
PGEM
pg/mL
1000
100
Control
Staurosporine
1 μM
Staurosporine
10 μM

Figure 6.9

Discussion.

The results of these experiments confirm the stimulatory effect of amniotic fluid obtained at spontaneous labour and elective caesarean section on PGE_2 production by amnion, as demonstrated by other investigators (Dowling et al, 1991; Cohen et al, 1985). Spontaneous labour amniotic fluid promoted significantly greater prostaglandin production compared with caesarean section fluid suggesting the presence of either increased stimulatory activity or decreased inhibitory activity in association with labour. In addition, spontaneous labour amniotic fluid significantly stimulated EM and FM production in chorion, reflecting increased prostaglandin synthesis within this tissue, which is in keeping with our previous findings (chapter five).

Prostaglandin synthesis is regulated at a number of points. Protein kinase C, by acting on phospholipase A_2 (PLA_2), can stimulate the direct release of substrate arachidonic acid from phosphatidylethanolamine. This is supported by the finding that the PLA_2 inhibitor quinacrine will prevent phorbol ester-induced stimulation of prostaglandin synthesis, even in the presence of exogenous arachidonic acid (Sander and Myatt, 1990). Alternatively, PKC can increase arachidonic acid concentrations indirectly at the level of phospholipase C, which mediates release of diacylglycerol (DAG) from phosphatidylinositol, that is subsequently metabolised to arachidonic acid. The DAG released serves to further enhance PKC activity. Via both of these mechanisms PKC increases availability of arachidonic acid for conversion to the prostaglandin endoperoxides, PGG_2 and PGH_2, a reaction which is dependent on the enzyme COX. In addition to its effect on substrate release, PKC is also able to stimulate transcription and translation, resulting in *de novo* synthesis of COX enzyme. This is demonstrated by the finding that new protein and messenger RNA synthesis are essential for the increased prostaglandin synthesis seen in response to phorbol esters, known

stimulators of PKC, and that irreversible inhibition of COX by acetylsalicylic acid pretreatment does not diminish their stimulatory effect (Zakar and Olson, 1988).

Stimulatory factors in amniotic fluid could therefore increase prostaglandin production by a direct effect on PKC, resulting in increased substrate release and COX synthesis. Alternatively, the site of action may be at the level of the phospholipases, again leading to increased substrate availability. The DAG produced in these reactions could then have a secondary effect on PKC, and thus COX synthesis.

We employed staurosporine (a PKC inhibitor) to investigate a possible role for PKC in the increased prostaglandin production by amnion seen in response to amniotic fluid. Phospholipid-dependent PKC activity has been demonstrated in human amnion (Okazaki et al, 1984), and activation of PKC has been associated with stimulation of prostaglandin synthesis in the initiation and maintenance of parturition (Olson et al, 1990). Known activators of PKC include phorbol esters, in particular 12-O-tetradeconylphorbol-13-acetate (TPA), and they have a stimulatory effect on prostaglandin production by amnion (Zakar and Olson, 1988; Lytton and Mitchell, 1988). This effect can be inhibited by a number of PKC inhibitors including staurosporine (Sander and Myatt, 1990; Zakar and Olson, 1992). In our experiments the addition of staurosporine to the amnion cultures resulted in significant inhibition of basal prostaglandin synthesis, and inhibition of amniotic fluid-stimulated prostaglandin synthesis, both spontaneous labour and elective section. The finding of decreased basal prostaglandin synthesis suggests that in our amnion cultures PKC has a role in maintaining basal prostaglandin production, but is not the sole mechanism involved since prostaglandin synthesis was not abolished by staurosporine. This is in contrast to the findings of other investigators who showed that staurosporine had no significant effect on unstimulated prostaglandin production (Zakar and Olson, 1992; Mitchell et

al, 1994), and may reflect differences in the duration of the cell cultures as the cells ability to respond changes with time (Gibb et al, 1990).

The inhibition of amniotic fluid-stimulated prostaglandin synthesis by staurosporine confirms that PKC is involved in this process. However, spontaneous labour amniotic fluid continued to stimulate significantly greater prostaglandin production than caesarean section amniotic fluid, or basal output suggesting an additional mechanism/s in this process. The stimulatory effect of caesarean section amniotic fluid was completely abolished by staurosporine 10 μM, reducing prostaglandin production to similar levels as basal output under the same conditions. This could be due either to the presence of lower concentrations of stimulatory factors in amniotic fluid prior to the onset of labour, or to the absence of stimulatory factors necessary to activate alternative or additional mechanisms of prostaglandin synthesis.

Cycloheximide significantly inhibited baseline prostaglandin production in our cultures, indicating that new protein synthesis, is occurring in the amnion cultures at this time. This is in keeping with the findings of Zakar and Olson (1992), and confirms that the PGE_2 accumulating in the medium was produced *de novo* in a protein synthesis dependent manner. Cycloheximide consistently inhibited the stimulatory effect of amniotic fluid, spontaneous and elective section, on prostaglandin production, indicating that new protein synthesis is necessary in this process. This suggests that COX production may be reduced. Since the inhibitory effect of cyclohexamide on amniotic fluid stimulated prostaglandin production is greater than its effect on baseline PG production, it may be production of COX-2 that is being inhibited. Amniotic fluid may therefore contain a factor which increases mRNA for COX-2.

Actinomycin D, an RNA synthesis inhibitor, significantly inhibited basal prostaglandin production by amnion suggesting that continuous RNA synthesis was contributing to prostaglandin output by these cell cultures at that time point in the culture system. This is in contrast to the findings of Zakar and Olson (1992), who found that unstimulated prostaglandin output was not affected by actinomycin D. Our findings with regard to the effect of actinomycin D on amniotic fluid-stimulated prostaglandin production were inconsistent between the two different cultures. However, within each culture the trends were the same suggesting that culture variability may play a part in this inconsistency. The effect of actinomycin D is known to be dose-dependent (Lytton and Mitchell, 1988; Zakar and Olson, 1992), with low concentrations leading to stimulation of phorbol ester-induced prostaglandin production, and high concentrations abolishing this effect, but at the doses employed in our experiments we would have expected inhibition if RNA synthesis was essential for the effect seen with amniotic fluid, and this reached significance in one culture. The finding that new protein synthesis, and perhaps mRNA production, are required for the stimulatory effect of amniotic fluid on prostaglandin production suggests that this effect is mediated by new enzyme synthesis, and it may be production of cyclooxygenase that is being inhibited by these agents. However, it cannot be assumed that cycloheximide and actinomycin D are inhibiting transcription and translation processes specific to amniotic fluid since these agents will inhibit all such mechanisms within cell cultures.

Again, the finding that spontaneous labour amniotic fluid continued to stimulate significantly more prostaglandin production by cells treated with cycloheximide than caesarean section amniotic fluid suggests the presence of additional factors in spontaneous labour fluid which may increase prostaglandin production by stimulating alternative pathways. Or, this may simply reflect an increased concentration of a single stimulatory factor in association with labour. Alternatively, caesarean section fluid may promote translation of existing mRNA leading to increased protein

synthesis, presumably COX-2, whereas spontaneous labour amniotic fluid may stimulate both transcription and translation resulting in greater total protein production. Futhermore, fluid from spontaneous labour may have a greater effect on substrate availability (PLA_2 / PKC) which would promote increased prostaglandin production without increasing the amount of COX in the cell.

We have demonstrated that in these amnion cultures the stimulatory effect of amniotic fluid on prostaglandin production is mediated, at least in part, by protein kinase C activation and new protein synthesis. In our chorion cultures, the stimulatory effects of amniotic fluid, spontaneous labour and elective section, were inhibited by staurosporine and cyclohexamide implicating PKC activity and new protein synthesis as seen in the amnion cultures. New mRNA appears to be involved in the stimulatory effect of spontaneous labour amniotic fluid. Thus, there is probably a dual mechanism operating, one increasing enzyme production and one increasing substrate availability, and PKC could be responsible for both.

In addition to PKC, tyrosine kinase has a pivotal role in controlling signalling within the cell, and hence influencing cellular metabolism, the cell cycle and growth. The receptors for a number of growth factors including insulin and the cytokine epidermal growth factor (EGF) have been shown to possess tyrosine kinase stimulatory activity (White, 1991). Activation of tyrosine kinase systems results in concommitant activation of several different protein kinases, and it is becoming increasingly apparent that there is a complex cascade of cell signalling involving significant interplay between various transduction pathways (Gupta, 1993). For example, it has been suggested that activation of PKC by TPA may result in activation, either directly or indirectly, of a number of tyrosine specific kinases (Gilmore and Martin, 1983). We investigated a potential role for tyrosine kinase in the amniotic fluid-stimulated increase in prostaglandin production by employing the tyrosine kinase inhibitor genestein in our

cell cultures. The effect of spontaneous labour amniotic fluid was consistently inhibited in both amnion and chorion cell cultures. This finding demonstrates that activation of tyrosine kinase, either directly or indirectly, is occurring in response to amniotic fluid, and confirms that signal tranduction processes are important in mediating amniotic fluid's stimulatory effect.

We have not attempted to characterise regulatory substances in amniotic fluid and therefore can only speculate as to what these agents might be. There is considerable interest in the role of cytokines in the process of parturition, which stems from investigations into preterm labour associated with intraamniotic infection. In this setting significantly higher concentrations of inflammatory cytokines such as IL-1, IL-6 and TNFα are found in the amniotic fluid compared with women in preterm labour in the absence of infection (Romero et al, 1989b; Romero et al, 1989e; Romero et al, 1990b). Cytokines are also found in higher concentrations in amniotic fluid obtained from spontaneous labour at term compared with term elective caesarean section, implying a role for them in the onset of labour at this time. Indeed, it has been suggested that parturition is an inflammatory-mediated process, similar to the events taking place during cervical ripening. Such agents could be involved in the regulatory process of prostaglandin production through PKC and tyrosine kinase stimulation, and stimulation of transcription and translation for essential enzymes such as cyclooxygenase.

Chorion and decidua are able to synthesise a number of cytokines including IL-1, IL-6, IL-8 and TNFα (Romero et al, 1989c; Dudley et al, 1992; Casey et al, 1989a), and these cytokines are capable of regulating their own production and that of each other (Mitchell et al, 1993a). In addition, it has been demonstrated that cytokines can stimulate prostaglandin production by amnion and chorion (Mitchell et al, 1991b; Kent et al, 1993; Romero et al, 1989d; Lundin-Schiller and Mitchell, 1991b) thus facilitating

myometrial contractility. There is therefore potential for a complex network of events at the choriodecidual interface which could in turn, via the amniotic fluid, influence the timing of parturition.

One attractive cytokine for a role in the regulation of parturition is EGF. This is a single chain polypeptide which may in part be derived from the fetal kidney (Haigh et al, 1989) supporting the hypothesis of fetal signalling in the control of labour. EGF/TGFα activity, as measured by radioreceptor assay, has been shown to be increased four fold in amniotic fluid collected from women in spontaneous labour (Romero et al, 1989f). EGF binding sites have been demonstrated in fetal membranes and decidua (Rao et al, 1984), and EGF is capable of stimulating PGE_2 production by human amnion (Mitchell, 1987) and chorion (Lundin-Schiller and Mitchell, 1991a). The mechanisms involved in the stimulatory actions of cytokines on prostaglandin production within the intrauterine tissues have been investigated in a number of studies. For example, phorbol myristoyl acetate (PMA) will potentiate the action of EGF in stimulating prostaglandin production by amnion cells (Kniss et al, 1990) and this EGF-stimulated prostaglandin production is inhibited by staurosporine. However, down regulation of PKC by prolonged exposure to phorbol ester did not reduce the stimulatory effect of EGF. These findings suggest that PKC has a role in modulating the action of this cytokine, but that PKC-independent pathways also exist whereby EGF exerts its effect.

The function of PKC in fetal membrane prostaglandin production is, however, complex as illustrated by the fact that the stimulatory effect of IL-1β on amnion PGE_2 production can be attenuated by down regulation of PKC, and in contrast staurosporine has been shown to enhance the effects of IL-1β (Mitchell et al, 1994). It has been suggested that these contrasting results may be due to interaction at different sites of the PKC molecule. An attractive alternative explanation is that the different

isoforms of PKC, which are now known to exist (Nishizuka, 1988), respond to activators in a differential manner.

Mitchell et al (1993b) have demonstrated that IL-1β's stimulatory effect on amnion PGE_2 production does not occur until after two hours of incubation, implying an induction process. This effect is dependent on new protein and RNA synthesis since it is inhibited by cycloheximide and actinomycin D (at concentrations ≥ 1 μg and 10 μg/mL respectively). They also showed in these experiments that IL-1β treated cells recovered more quickly from pretreatment with acetylsalicylic acid than controls, suggesting that the action of IL-1β involves induction of COX protein. Further investigation employing Western blot analysis confirmed an increase in COX protein, and Northern blot analysis demonstrated the presence of mRNA for an inducible form of COX, now known as COX-2.

Similar experiments have been conducted on chorion cell cultures (Pollard et al, 1993). The stimulatory actions of IL-1β, EGF and TNFα on PGE_2 production in this tissue are all inhibited by treatment with actinomycin D and cycloheximide, supporting a role for new protein and mRNA synthesis. Again, recovery from acetylsalicylic acid pretreatment was quicker in response to all three cytokines, suggesting that *de novo* synthesis of COX is involved in mediating their effect. These investigators also found that addition of exogenous arachidonic acid enhanced the stimulatory effect of EGF, IL-1β and TNFα on prostaglandin production, and concluded therefore that these cytokines act to enhance conversion of arachidonic acid to prostaglandins, rather than promote arachidonic acid release via phospholipase activation.

The experiments described in this chapter indicate that PKC and new protein synthesis are important in the stimulatory effect of amniotic fluid and this would be compatible with inflammatory mediators such as cytokines being central to this process.

Chapter Seven

Recombinant Human Relaxin as a Cervical Ripening Agent

Introduction.

In order that induction of labour replicates the normal physiological process of parturition as closely as possible, the question of cervical ripening must be addressed. The success of labour induction is influenced by the state of the cervix. Induction of labour in the presence of an unripe cervix is associated with an increase in both maternal and neonatal morbidity (Calder, 1979). Cervical ripening occurs during the phase known as pre-labour. Its exact mechanism remains unclear but the process results in structural changes within the cervix making it more compliant. These include a decrease in collagen concentration, an increase in water content and an alteration in proteoglycan/glycosaminoglycan ratio within the tissue. It is this process that we are striving to mimic when ripening the cervix pharmacologically. The ideal cervical ripening agent should have a selective effect on the cervix without any additional effect on uterine contractility, and in theory the polypeptide hormone relaxin would meet these requirements.

Relaxin was first identified by Hisaw (1926) who demonstrated its capacity to promote separation of the pubic symphysis in guinea-pigs. Relaxin facilitates connective tissue remodelling, and in some animals also inhibits myometrial contractility. In most species, cervical ripening prior to the onset of labour is associated with an increase in serum relaxin concentrations. However, this is not the case in humans and the exact role of this hormone in human parturition remains uncertain. Early clinical trials (Eisenberg, 1957; Decker et al, 1958) employing porcine relaxin for cervical ripening in human subjects reported conflicting results which probably reflected the impurity of the agent at that time. The subsequent purification of porcine relaxin (Sherwood and O'Byrne, 1974) led to renewed interest in this hormone. A number of studies showed that porcine relaxin had some therapeutic benefit as a cervical ripening agent in women (MacLennan et al, 1980; Evans et al, 1983), despite the primary peptide structure of

porcine relaxin having only about 50% homology with that of human relaxin. The development of recombinant human relaxin by Genentech Inc. (San Francisco, CA, USA) has provided an agent that could potentially have superior cervical ripening properties in women and has stimulated further research in this field.

Phase I studies conducted by Genentech demonstrated that recombinant human relaxin was safe and not associated with serious adverse effects, and that there was no maternal development of antibody to rhRIx following treatment (Spanski and Perlman, 1991; Spanski and Perlman, 1991). Phase II studies were therefore initiated in Australia and the UK. Presented in this chapter are the results of the UK double-blind, three-centre study investigating the effect of recombinant human relaxin, administered as an intravaginal gel, on cervical ripening in pregnant women at term with an unfavourable cervix.

Subjects and methods.

Ethical approval for this study was obtained from the local ethics committees of the three participating centres.

Ninety-six women, comprising nulliparous and parous patients, were recruited to the study. All women had a singleton pregnancy of at least 37 weeks gestation with a cephalic presentation and a modified Bishop score (Calder 1974) of four or less. Women with a uterine scar, ruptured membranes or evidence of placental abruption or placenta praevia were not recruited to the study. Other exclusion criteria were significant systemic disease, recent ingestion of aspirin or other non-steroidal anti-inflammatory drugs, fetal malformation, growth retardation or macrosomia and oligo- or polyhydramnios. The decision to induce labour was made by the woman's

attending obstetrician and written informed consent was obtained prior to recruitment. The most common indications for induction were pregnancy induced hypertension and prolonged pregnancy.

The study involved a randomised, double-blind comparison of four treatment regimens: 0 (placebo), 1, 2 or 4 mg of recombinant human relaxin (rhRIx) in a gel vehicle. The randomisation sequence was generated in blocks of four by the Genentech Biostatistical Department and the code was stratified according to parity. A randomisation list accompanied each drug shipment. Primiparous women were assigned sequential numbers starting from the top of the list, and multiparous women were assigned sequential numbers starting from the bottom of the list. The study medication was supplied by Genentech Inc. as a two-part administration set: a stoppered 20 cc glass vial of lyophilized rhRIx or placebo and a 5 cc syringe of gel. The vial contained 0 mg or 12 mg rhRIx in isotonic citrate buffer. The syringe delivered 3.0 mL of sterile 4% methylcellulose gel in isotonic citrate buffer. The active doses were made by reconstituting 12 mg of lyophilized study medication in either 12, 6 or 3 mL of sterile water and combining this with the fixed 3 mL volume methylcellulose gel.

Women were admitted to hospital in the afternoon prior to the day of induction. A medical and obstetric history was taken and a general examination performed. Fetal weight and liquor volume were estimated by ultrasound scan and a cardiotocograph performed. Blood was taken for haematology, coagulation profile, biochemistry, serum relaxin level and serum antibody to relaxin. Upon randomisation, the study medication was reconstituted using sterile water by one of the hospital pharmacy staff and delivered to the labour ward. The baseline modified Bishop score was recorded and the study gel administered intravaginally to the posterior fornix that evening. The woman remained recumbent for 1 hour following gel application. Blood pressure,

pulse, respiration rate, uterine activity and fetal heart rate were monitored hourly for four hours post-treatment, and then every four hours for at least 24 hours or until delivery. Observations were suspended overnight if the patient was asleep. Blood was taken at 1, 4, 15 and 24 hours after gel administration for serum relaxin concentrations.

The following morning, at approximately 15 hours post-treatment, a vaginal examination was performed by the same investigator and the modified Bishop score recorded after which prostaglandin E_2 (PGE_2) gel 2 mg was instilled intravaginally. Further PGE_2 gel, 1 or 2 mg, was given after a six hour interval as required. Amniotomy was performed when the cervix was at least 3 cm dilated and fully effaced and oxytocin administered according to hospital protocol. Uterine activity and fetal heart rate were monitored continuously throughout labour. If labour occurred prior to the planned 15 hour assessment, the modified Bishop score was recorded at the onset of labour and the patient managed as above. The onset of labour was defined as the onset of regular painful contractions with evidence of progressive cervical dilatation of the cervix. For women delivered by caesarean section prior to the second stage of labour, the length of the first stage was taken to be the total length of labour.

Umbilical cord blood was taken at delivery for assay of relaxin concentrations and blood gas analysis, and maternal blood was collected to measure serum relaxin concentrations. Apgar scores were recorded at 1 and 5 minutes post-delivery. Twenty four hours after delivery, maternal blood was collected for repeat haematology, coagulation and routine biochemistry profiles, and serum relaxin concentration measurement. A general physical examination was also performed. Women were reviewed six weeks post-delivery when serum relaxin conccentrations were measured and any postnatal problems documented.

The primary outcome measure was the change in modified Bishop score between baseline and 15 hours post-treatment. Secondary outcome measures included duration of first and second stage of labour; prevalence of "spontaneous" labour and caesarean section, need for oxytocin and total PGE_2 requirement. Maternal safety measures included vital signs, haematology and biochemistry screening. Fetal safety measures included stillbirth, neonatal death, fetal heart rate disturbances, Apgar scores, cord blood gases, need for resuscitation and incidence of neonatal morbidity.

Sample Size and Power Consideration.

Ninety-six patients were recruited to the study with 24 patients in each of the four treatment groups. The primary efficacy variable, change in modified Bishop score, was first to be analysed by ANOVA to take account of any effect of dose influencing modified Bishop score. If a significant effect was demonstrated subsequent pairwise comparisons were to have been performed. The determination of sample size was based on such pairwise comparisons. Assuming that the standard deviation and the between-treatment difference with respect to the change in cervical score are equal, a two-sided t-test with a significance level of 1.67% will have 84% power to detect such a difference.

Statistical Analysis.

All data were recorded on standardised case record forms and analysed at Genentech Inc. in California. Analysis of variance tests were performed on all continuous variables, and Cochran Mantel-Haenszel tests employed for discrete variables.

Results.

Randomisation of patients to the four treatment groups was as follows: placebo - 23, 1 mg - 23, 2 mg - 25 and 4 mg - 25. There were no statistically significant differences in the characteristics of the women in the four groups (Table 7.1).

The mean changes in modified Bishop score were 1.64 for placebo, 1.35 for 1 mg relaxin, 1.76 for 2 mg relaxin and 1.32 for 4 mg relaxin. There was no statistically significant difference in the increase in modified Bishop score between the four groups (Table 7.2). In addition, there was no significant difference between the four groups when nulliparae and parous women were analysed separately (data not shown). The length of the first and second stages of labour was similar in all treatment groups. There were no differences in the time from treatment to first stage of labour or from treatment to delivery between the groups (Table 7.2). Excluding the patients delivered by caesarean section from the analysis did not alter the results for any of the above variables (data not shown).

The majority of women required induction of labour 15 hours after study medication and only a few subjects laboured before this time (Table 7.3). The use of PGE_2 and oxytocin is also illustrated in table 7.3. There was no statistical difference in requirement for either drug between groups, and the doses employed were similar in each treatment group. There was no significant difference in the mode of delivery between the groups with the majority of women achieving a spontaneous vaginal delivery (Table 7.4). The overall caesarean section rate for the study was approximately 20% and the apparent increase in section rate in the 2 mg group did not reach statistical significance. Again, there was no statistical difference in the variables displayed in tables 7.3 and 7.4 when primigravid and parous women were analysed

separately, or when women delivered by caesarean section were excluded from the analysis (data not shown).

Maternal and neonatal safety outcomes.

There was no significant difference in pre- and post-treatment measurements of pulse and respirations between the four groups. There was a tendency to lower blood pressures in the treatment groups when compared with placebo and this was statistically significant for diastolic blood pressure: placebo-83 mm Hg; 1, 2 and 4 mg rhRIx -77, -77 and 70 mm Hg respectively ($p \leq 0.012$). This effect was seen over the first 14 hours following the start of treatment. Haematological and biochemical profiles were within the normal range for pregnancy. There was no association between other maternal outcome measures such as postpartum haemorrhage, infection and urinary retention and treatment group.

There were significantly higher fetal heart rates in the treatment groups: placebo -133 beats per minute (bpm); 1, 2 and 4 mg rhRIx -136, -141 and 137 bpm respectively ($p \leq 0.011$). This effect was evident for approximately 24 hours post-treatment. There were no significant differences in 1 and 5 minute Apgar scores, or cord blood gas results between the groups. Neonatal outcome measures such as meconium stained amniotic fluid, hypoglycaemia and hyperbilirubinaemia occurred with the same frequency in all treatment groups. There were no stillbirths or neonatal deaths in the study.

Relaxin concentrations.

Maternal serum relaxin concentrations were assayed by Genentech. Concentrations following intravaginal administration of 1, 2 and 4 mg rhRIx were no different from the endogenous relaxin levels measured in the placebo group (Fig. 7.1). There was no correlation between baseline serum relaxin concentration and baseline modified Bishop score, or maximum serum relaxin concentration and change in modified Bishop score. The majority of cord blood relaxin concentrations were below the level of detection of the assay irrespective of treatment group. Details of the relaxin assay are "in house" at Genentech.

	Treatment Group				
Characteristic	Placebo (n=23)	1 mg (n=23)	2 mg (n=25)	4 mg (n=25)	p value
Age (years)	27.0	26.8	26.7	25.8	
	(21.3-32.7)	(21.3-32.3)	(22.1-31.3)	(21.4-30.2)	0.796
Modified	2.9	3.0	2.8	2.5	
Bishop Score	(2.0-3.8)	(2.3-3.7)	(1.9-3.7)	(1.4-3.6)	0.076
Gestation (weeks)	40.0	39.6	39.9	40.1	
	(38.5-41.5)	(38.2-41.0)	(38.8-41.0)	(38.9-41.3)	0.694
Parity					
0	18	20	22	19	
1+	5	3	3	6	0.581

Table 7.1. Subject characteristics at recruitment, by treatment group (mean, SD).

Age and gestation analysed by ANOVA; parity and baseline modified Bishop score analysed by Cochran Mantel-Haenszel.

	Treatment Group				
	Placebo	1 mg	2 mg	4 mg	p value
Change in Bishop Score	1.64	1.35	1.76	1.32	
	(1.0-2.3)	(0.7-2.0)	(0.5-3.0)	(0.8-1.9)	0.853
Duration of First Stage (hours)	4.9	5.0	5.3	6.5	
	(3.9-5.9)	(3.7-6.2)	(4.1-6.5)	(5.1-7.9)	0.222
Duration of Second Stage (hours)	0.7	1.3	1.0	1.1	
	(0.4-1.1)	(0.8-1.7)	(0.5-1.5)	(0.6-1.5)	0.387
Total Duration of Labour (hours)	5.6	6.2	6.3	7.6	
	(4.6-6.6)	(4.7-7.8)	(4.9-7.7)	(5.9-9.2)	0.286
Treatment-to-First Stage (hours)	22.3	23.7	26.9	23.1	
	(19.2-25.3)	(21.0-26.3)	(22.3-31.6)	(20.2-25.9)	0.202
Treatment-to Delivery (hours)	28.0	29.9	39.3	36.7	
	(24.8-31.2)	(26.7-33.2)	(26.4-52.2)	(23.8-49.6)	0.306

Table 7.2. Labour characteristics, by treatment group (mean, 95% CI).

	Treatment Group				
	Placebo (n=23)	1 mg (n=23)	2 mg (n=25)	4 mg (n=25)	p value
"Spontaneous" Labour				*	
No	21	22	20	22	0.314
Yes	2	1	5	2	
Patients requiring PGE_2	18	18	21	20*	0.928
Total PGE_2 dose (mg)	2.1	2.1	2.6	2.3	
	(1.5-2.6)	(1.5-2.6)	(2.0-3.1)	(1.8-2.8)	0.553
Patients requiring Oxytocin	10	13	12	13	0.916
Total Oxytocin dose (U)	2.4	2.7	2.6	2.4	
	(0.4-4.4)	(1.2-4.1)	(0.8-4.5)	(1.1-3.7)	0.993

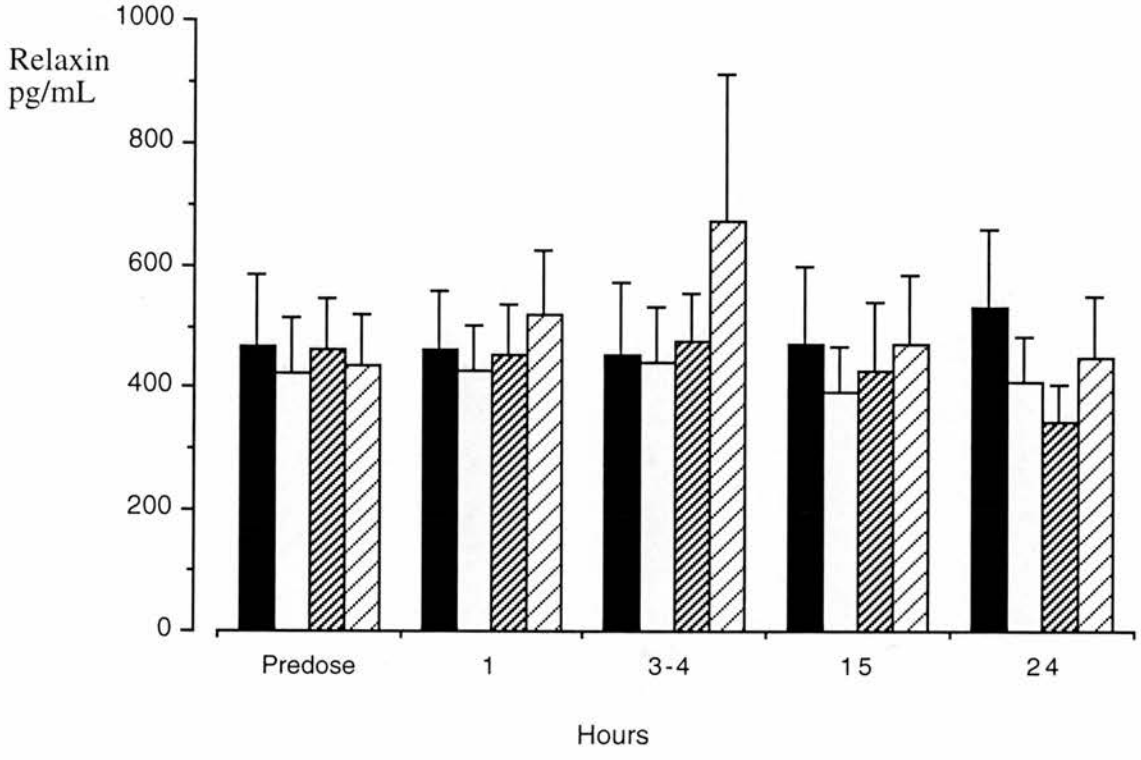
Table 7.3. Oxytocin and PGE_2 requirements, by treatment group (mean, 95% CI)
Analsyed by ANOVA

	Treatment Group				
	Placebo (n=23)	1 mg (n=23)	2 mg (n=25)	4 mg (n=25)	p value
Mode of Delivery:	*				
Vaginal	16	14	13	16	0.446
Operative Vaginal	2	6	4	5	
Caesarean Section	4	3	8	4	

Table 7.4. Delivery Characteristics by treatment group (mean, 95% CI)

* Data missing for one subject in this group.

Analysed by Cochran Mantel-Haenszel test.


Relaxin
pg/mL
1000
800
600
400
200
0
Predose
1
3-4
15
24
Hours

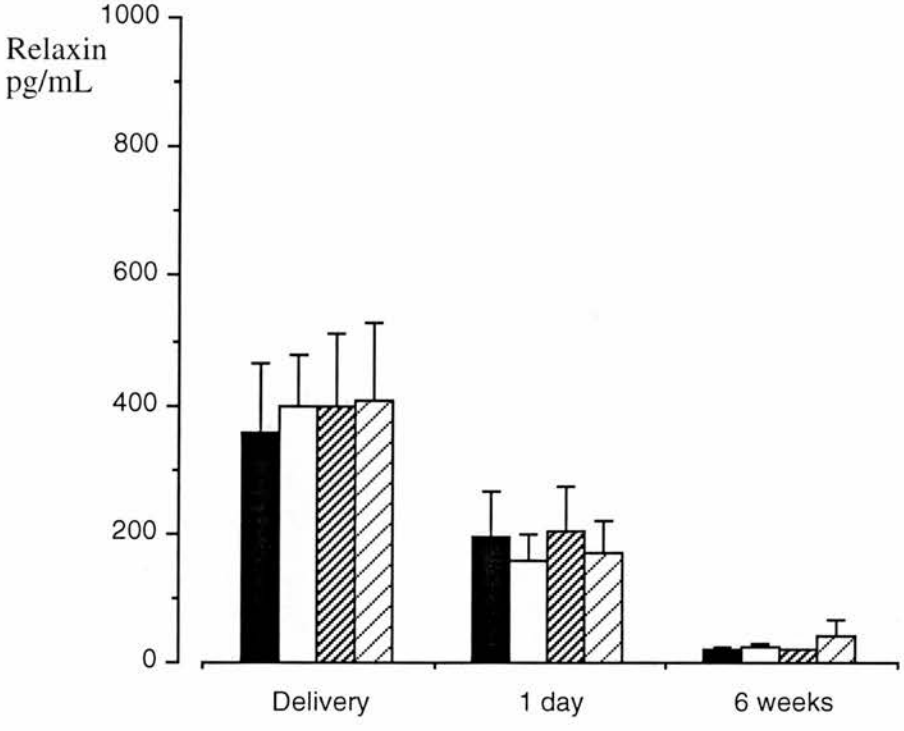

Relaxin
pg/mL
1000
800
600
400
200
0
Delivery
1 day
6 weeks

Figure 7.1

Discussion.

A precise role for relaxin in the physiology of human parturition has yet to be defined. In a number of animals (eg. pig and rat), the onset of labour is clearly preceded by a surge in serum relaxin concentrations and this appears to facilitate connective tissue remodelling in the reproductive tract. In human pregnancy serum relaxin concentrations peak at approximately ten weeks gestation and thereafter concentrations fall progressively towards term (Bell et al, 1987). Labour itself is not associated with any further change in relaxin concentrations (Bell et al, 1987). If relaxin plays a key role in cervical ripening it would be during the time of pre-labour that changes in serum relaxin concentrations might occur.

The corpus luteum is the primary source of relaxin during pregnancy, although extra-ovarian sites of production have been identified (Sakbun et al, 1990). The fact that relaxin is not a prerequisite for cervical dilatation has been demonstrated by the successful induction of labour in a woman with premature ovarian failure in whom serum relaxin levels were unrecordable (Eddie et al, 1990). This finding also highlights the fact that relaxin production by other tissues is very small compared with ovarian production.

This study did not show any therapeutic effect of recombinant human relaxin on cervical ripening when administered as an intravaginal gel. There was no significant difference in mean change in modified Bishop score following treatment with 1, 2 or 4 mg of rhRIx compared with placebo. This is not surprising since pre- and post-treatment serum relaxin concentrations were the same in the placebo and active groups suggesting that relaxin was not absorbed from the preparation when administered vaginally, or that the dosages were too small. There was no association between endogenous serum relaxin levels and baseline modified Bishop score, or maximum

serum relaxin concentrations and change in modified Bishop score. In addition, there was no change in endogenous levels throughout induction, labour and delivery which confirms previous findings. RhRIx appears to be safe and not associated with any clinically significant side effects. In view of the lack of evidence for absorption of rhRIx, which is reflected in the absence of clinical effect, the changes in maternal diastolic blood pressure and fetal heart rate, which were small and clinically irrelevant, may well have been spurious.

These findings are in keeping with a smaller study (Bell et al, 1993) employing recombinant human relaxin in a dose of 1.5 mg which also failed to demonstrate a significant effect on cervical ripening. Similarly, this dose of recombinant human relaxin was not associated with any adverse maternal or fetal complications. One explanation for a role for relaxin in cervical ripening without any parallel increase in circulating relaxin concentrations would be if it were acting at receptor level. However, to date, a receptor for relaxin in humans has not been identified.

The reduction in cervical collagen concentration necessary for cervical ripening can, in part, be explained by an increase in enzymatic collagen degradation. Labour is associated with an increase in circulating collagenase levels (Rajabi et al, 1985) and collagenase activity within cervical tissue. It has been reported that relaxin increases collagenase activity (von Maillot et al, 1977). Relaxin receptors have been identified on human fibroblasts (McMurty et al, 1980), which along with leucocytes are capable of collagenase production. A number of clinical trials have demonstrated a cervical ripening effect of porcine relaxin in pregnant women (MacLennan et al, 1980; Evans et al, 1983). However, these trials were small and the patients were of mixed parity. In addition, in one study (MacLennan et al, 1980), patients in the treatment group tended towards higher baseline Bishop scores limiting the conclusions that can be drawn. Cervical ripening has been likened to an inflammatory response (Liggins, 1981) and

one potential mechanism whereby porcine relaxin, although purified, could stimulate cervical ripening is by provoking an immunological reaction resulting in neutrophil degranulation and release of collagenase perhaps mediated by interleukin-8, production of which has been demonstrated in the human cervix (Barclay et al, 1993).

The effect of relaxin on myometrial contractility in different species is varied. An inhibitory effect of purified porcine relaxin on the spontaneous contractility of non-pregnant human myometrium has been demonstrated *in vitro* (Szlachter et al, 1980). This effect has been confirmed on non-pregnant and pregnant myometrium from rats and pigs. However, porcine relaxin had little, if any, effect on spontaneous or induced, pregnant or non-pregnant, human myometrial contractility *in vitro* (MacLennan, 1983). More recently, human relaxin has been shown to have only a minor effect on human myometrial contractility in late pregnancy (MacLennan and Grant, 1991). The data for uterine activity in this clinical trial were not assessed, but as there was no difference in duration of labour or need for augmentation between groups it is likely that contractility was unaffected.

Prostaglandins are currently the most successful agents for ripening the cervix pharmacologically (Keirse, 1993) but they have the disadvantage of simultaneously stimulating uterine activity. Although theoretically relaxin meets the requirements for the ideal cervical ripening agent, that is ripening without contractility, this study has not demonstrated any cervical ripening effect of recombinant human relaxin. This may simply be due to incorrect choice of dosage. However, it seems more likely that the route of administration for this large polypeptide hormone was inappropriate. The intravenous route may be more suitable and deserves to be investigated before we can discard relaxin as a ripening agent.

Chapter Eight

Mifepristone for Cervical Ripening and Labour Induction at Term

Introduction.

Until labour pregnancy is characterised by myometrial quiescence. It has been suggested that progesterone is the hormone responsible for this (Csapo, 1975). Csapo (1975) proposed that progesterone withdrawal converts the uterus from an inert state to one of increased responsiveness to both endogenous and exogenous uterotonic agents. His suggestion that progesterone withdrawal also induces release of endogenous myometrial stimulants is supported by the finding that, in a number of species including rat, rabbit and sheep, progesterone withdrawal is the signal for the onset of parturition. However, a decline in peripheral circulating progesterone concentrations cannot be demonstrated in humans prior to the onset of labour. Csapo believed that where the placenta is the major source of progesterone as pregnancy advances, peripheral progesterone concentrations do not accurately reflect progesterone concentrations within the uterine target tissues. It is clear from studies investigating the efficacy of the progesterone antagonist mifepristone (RU 486) that progesterone does play an integral role in the timing of labour onset in "progesterone-independent" species such as humans.

Mifepristone, a potent antagonist of progesterone and glucocorticoid action (Spitz and Bardin, 1993), was first synthesised in 1981 by Roussel-Uclaf (Romainville, France). It is an orally active drug with a 70% absorption rate, and 40% bioavailability after first pass metabolism through the liver (Van Look and Bygdeman, 1989). It is protein-bound in the serum and has a half-life of approximately 20 hours. Mifepristone acts at the level of the progesterone receptor. The progesterone receptor, located in the cell nucleus, undergoes configurational changes when progesterone binds to it (Tsai and O'Malley, 1994) The resulting receptor complex can bind to the promoter region of progesterone-responsive genes and increase the transcription rate of genes responsible for mediating progesterone-dependent effects. Alternatively, when

mifepristone binds to the progesterone receptor, the resulting complex renders the DNA-bound receptors in the progesterone responsive genes transcriptionally inactive, interrupting the effects of progesterone. Based on this mode of action, antiprogestins such as mifepristone have a number of clinical applications, including early pregnancy termination, induction of labour in the second and third trimesters, and postcoital and prophylactic contraception (Ulmann et al, 1995).

Mifepristone will induce vaginal bleeding in virtually all women when administered in the early weeks of pregnancy, but its ability to induce complete abortion falls from 100% when given in the prenidatory stage of pregnancy (Glasier et al, 1992) to <40 % at greater than 56 days of amenorrhoea (Van Look and Bygdeman 1989), ie. the rate of complete abortion is inversely related to the gestation of pregnancy. The introduction of "combination" therapy, following the important observation that mifepristone increases the sensitivity of the uterus to prostaglandins (Bygdeman and Swahn, 1985), has led to a complete abortion rate of 95% in women ≤63 days gestation treated with mifepristone 600 mg and gemeprost 1 mg 48 hours later (UK Multicentre Trial, 1990).

Mifepristone's use is not confined to the first trimester of pregnancy. In the second trimester, mifepristone pretreatment will significantly reduce the induction-to-abortion interval compared with prostaglandin therapy alone, and will reduce the dose of prostaglandin required to effect abortion (Urquhart and Templeton, 1990; Rodger and Baird, 1990). It is also successful in inducing abortion in the presence of intrauterine fetal death when administered alone (Cabrol et al, 1990).

Mifepristone's mechanism of action is two-fold. Firstly, it increases myometrial responsiveness to oxytocic agents as a result of increased gap junction formation (Garfield and Beier, 1989) and possibly a negative effect on nitric-oxide dependent

myometrial relaxation (Yallampalli et al, 1993). Secondly, it promotes cervical ripening. Evidence for this is provided indirectly by the shortened induction-delivery interval in women undergoing medical termination of pregnancy who are pretreated with mifepristone, and directly by studies demonstrating the reduction in force necessary to dilate the cervix prior to surgical termination of pregnancy following mifepristone pretreatment (Durlot et al, 1988; Radestad et al, 1988). Because mifepristone induces cervical ripening and myometrial responsiveness without stimulating uterine contractions there are indications for its use as a cervical ripening agent in women with an unfavourable cervix at term, where uterine contractility in the face of an unripe cervix is inefficient and stressful for the fetus. In addition, mifepristone will induce cervical ripening in the non-pregnant cervix (Gupta and Johnson, 1990).

Frydman et al (1992), have investigated the role of mifepristone in cervical ripening at term and demonstrated that significantly more women went into "spontaneous" labour in the mifepristone treated group and that, of those undelivered at the end of the treatment interval, significantly fewer had an unfavourable cervix in the treatment group. Also, the total dose of oxytocin administered was significantly less in the mifepristone treated group. These findings were reproduced in a study of similar design investigating induction of labour in women with a single transverse caesarean section scar (Lelaidier et al, 1994). These studies provide encouraging information with regard to a role for mifepristone in induction of labour at term. In neither study was there maternal or neonatal complications. However, as mifepristone crosses the placenta (Hill et al, 1990), and has an antiglucocorticoid effect it has the theoretical risks of hypotension and hypoglycaemia in the neonate. In view of this, larger trials, including dose-finding studies, are required to confirm the efficacy and safety of this agent, and the lowest dose at which it is effective. This chapter describes the first arm of one such dose-finding study.

The aim of this study was to compare mifepristone, in an oral dose of 50 mg, with placebo for induction of labour at term. Part II of the study (not discussed here) was to investigate a lower (20 mg) or higher (200 mg) dosage of mifepristone, depending on the results obtained with the 50 mg dose.

Subjects and Methods.

This was a double-blind, placebo-controlled trial carried out in a single centre in the UK. Local ethical approval was granted. A total of 50 patients were recruited, 25 subjects randomised to receive mifepristone 50 mg and 25 subjects to receive placebo. Primigravid women at term (≥ 37 completed weeks gestation) with a cephalic presentation, an indication for induction of labour and a modified Bishop score (Calder, 1979) of ≤4 were invited to take part in the study. Written informed consent was obtained prior to entry to the study.

The following were exclusion criteria for the study:

- Signs or symptoms of labour onset.
- An indication for delivery within 72 hours.
- Previous attempt to induce labour in this pregnancy.
- Signs of placental insufficiency.
- Contraindication to vaginal delivery.
- History of ruptured membranes.
- Concurrent illness: renal failure, hepatic disorder, adrenal insufficiency, diabetes mellitus, coagulation disorders.
- Treatment with corticosteroids, anticoagulants, non-steroidal anti-inflammatory agents.

Women were recruited from the hospital's antenatal clinics. The most common reason for inclusion in the study was prolonged pregnancy (gestation > 40 completed weeks): 72 % and 88 % in the mifepristone and placebo groups respectively. Pregnancy induced hypertension was the second most common reason for inclusion: 24 % and 16% in the mifepristone and placebo groups respectively.

Subjects attended the hospital 72 hours prior to the scheduled day of induction. At this visit a routine medical history, physical examination and biochemical and haematological screening were carried out. A cardiotocograph (CTG) was performed and the modified Bishop score recorded. Subjects were allocated a study number and the corresponding treatment was administered to the patients in the presence of the clinician. Pre-determined randomisation codes were generated by Roussel Laboratories Statistical Unit, and subjects were allocated a study number, and therefore treatment, in strict numerical order as they were entered into the trial. Subjects were then managed as outpatients, returning to hospital every 24 hours for repeat assessments of blood pressure, pulse, modified Bishop score, CTG and fetal movements. Subjects were asked to complete a fetal kick chart recording the length of time taken to feel ten fetal movements in each 24 hour period.

If labour occurred spontaneously within 72 hours of study medication subjects were managed according to normal hospital protocol. If labour was not established 72 hours following study treatment, the modified Bishop score was assessed and labour induced with prostaglandin gel, in an initial dose of 1 mg. If required, a repeat dose of prostaglandin gel (1 or 2 mg) was administered after a six hour interval. Amniotomy was performed when the cervix was 3 cm dilated and fully effaced, and oxytocin was administered intravenously four hours later if clinically indicated. Cardiotocography was performed continuously throughout labour.

Cord blood was taken at delivery for measurement of adrenocorticotrophin hormone (ACTH), cortisol and mifepristone concentrations, blood gases, pH and base excess, biochemistry and haematology. Birth weight and 1 and 5 minute Apgar scores were recorded. Neonatal blood glucose levels were measured with Dextrostix from heel stab samples at 1, 3 and 12 hours following delivery. Maternal blood was taken at 24-48 hours following delivery for measurement of haematological and biochemical indices. Clinical assessments of the mother and neonate were made at 24 and 48 hours, and one and four weeks post delivery.

For the ACTH and cortisol measurements 4 mL of cord blood was drawn into a heparinised tube, and placed in an ice bath. The sample was immediately centrifuged at 1500 g at 4 C for ten minutes and plasma separated into two dry tubes and frozen at -20 C pending assay. ACTH and cortisol concentrations were assayed at the Royal Infirmary, Glasgow and the Royal Infirmary, Edinburgh respectively. The mifepristone assays were carried out by the Department of Pharmacokinetics, Rousel Uclaf Laboratories, Compiegne, France.

The primary outcome measures for the study were the success or failure of labour induction and the degree of cervical ripening as measured by the modified Bishop score. Secondary outcome measures included time to membrane rupture, full dilatation and delivery; requirement for prostaglandin and oxytocin administration, including total dose; need for amniotomy and mode of delivery. Maternal and neonatal adverse events were recorded.

Statistical Analysis.

Continuous variables were analysed using ANOVA and categorical data were analysed using the Chi-squared test.

Results.

Fifty patients were recruited to the study and allocated to two treatment groups: mifepristone 50 mg (n=25) or placebo (n=25). The characteristics of the women enrolled are illustrated in table 8.1, and there were no statistically significant differences between the two groups apart from weight which was accounted for in the efficacy analysis.

The primary outcome measures were divided into the following three categories: total success, partial success and failure. Total success was defined as onset of labour or modified Bishop score of ≥ 6 within 72 hours of treatment administration without further intervention. Partial success was defined as onset of labour or modified Bishop score ≥ 6 within 72 hours of treatment administration with further intervention required. The results are summarised in table 8.2.

Characteristic	Treatment group		P
	Mifepristone 50 mg	Placebo	
Age (years)	25.8 (24.0 - 27.6)	26.5 (24.0 - 28.9)	0.647
Height (cm)	165 (162.6 - 167.0)	162 (159.3 - 164.7)	0.104
Weight (kg)	89.4 (82.2 - 96.7)	78.5 (73.3 - 83.6)	0.014
Gestation (weeks)	40.7 (40.4 - 41.0)	40.8 (40.6 - 41.0)	0.738
Modified Bishop score	3.5 (3.2 - 3.8)	3.2 (2.9 - 3.5)	0.132

Table 8.1. Subject characteristics by treatment group. All parameters analysed by ANOVA.
Values are given as mean (95% CI).

	Treatment group		
	Mifepristone 50 mg	Placebo	P
Total success	2	2	1.00
Partial success	10	4	0.059
Failure	13	19	0.077

Table 8.2. Primary outcome measure by treatment group. Values are given as n.

Only 2 subjects (8%) in each treatment group had a successful outcome without the need for further intervention. There was no significant difference for primary outcome in either of the three categories between the treatment groups. However, the number of subjects with a successful outcome after further intervention (partial success) was notably higher in the mifepristone group compared with placebo (40% versus 16% respectively).

There was no significant difference in the length of the first and second stages of labour between the two treatment groups (Table 8.3). The times from treatment to membrane rupture, treatment to onset of labour, and from treatment to delivery were also similar between the two groups (Table 8.3). Similar numbers of women in the mifepristone and placebo groups required treatment with prostaglandin gel, and the total dose administered was the same for the two groups. The number of women requiring oxytocin augmentation was also similar, and there was no significant difference in the total dose of oxytocin infused (Table 8.4).

There was no significant difference in the number of women achieving a spontaneous vertex delivery between the two groups (mifepristone 64%; placebo 44%), and the same was true for the instrumental delivery rate (mifepristone 28%; placebo 24%) (Table 8.4). However, the caesarean section rate was significantly lower in the mifepristone group: 8% versus 32%, $p<0.05$.

	Treatment group		
	Mifepristone 50 mg	Placebo	P
Duration of 1 st stage (h)	6.59 (4.9 - 8.3)	6.86 (5.6 - 8.1)	0.803
Duration of 2 nd stage (h)	0.99 (0.73 - 1.25)	1.56 (0.73 - 2.4)	0.140
Total duration of labour (h)	7.6 (5.75 - 9.4)	8.7 (7.6 - 9.7)	0.281
Treatment to membrane rupture (h)	69.6 (58.1 - 80.2)	75.7 (65.3 - 86.2)	0.357
Treatment to 1 st stage (h)	69.1 (58.1 - 80.2)	75.7 (65.3 - 86.2)	0.374
Treatment to delivery (h)	75.9 (64.7 - 87.1)	84.5 (74.0 - 95.0)	0.255

Table 8.3. Labour characteristics by treatment group. All parameters analysed by ANOVA. Values are give as mean (95% CI).

	Treatment group		
	Mifepristone 50 mg	Placebo	P
Women requiring PGE_2	17	20	0.333
Total PGE_2 dose (mg)	2.35 (1.95 - 2.76)	2.35 (1.94 - 2.76)	0.991
Women requiring oxytocin	8	12	0.248
Total oxytocin dose (U)	8.5 (3.3 - 13.7)	9.04 (4.8 - 13.3)	0.856
Mode of delivery:			
Vaginal	16	11	0.156
Operative vaginal	7	6	0.747
Caesarean section	2	8	0.034

Table 8.4. Delivery characteristics, oxytocin and PGE2 requirements, by treatment group. Values are given as n and mean (95% CI)

Maternal Safety

Study medication was well tolerated by the subjects. Maternal adverse events such as postpartum haemorrhage, anaemia, placental disorder, fever and abnormal labour were reported with a similar frequency between the two groups. All were considered by the clinical investigators to be mild or moderate in severity, and only one event, headache, was considered to be possibly related to treatment. There were no maternal deaths in the study. Haematological and biochemical parameters assessed ante- and postnatally were considered to be normal for pregnancy or the given clinical situation eg. anaemia following postpartum haemorrhage. There were no abnormal clinical findings at the 24 and 48 hours check ups, or the one and four week postnatal reviews.

Fetal Safety

Maternal assessment of fetal movement 24, 48 and 72 hours after treatment is illustrated in table 8.5. There was no significant difference in the results between the two groups.

Reporting of fetal adverse events was similar for the two treatment groups. Such events included acute fetal distress, bradycardia, fetal heart rate (FHR) abnormality, meconium stained amniotic fluid and fetal tachycardia. Three serious adverse events were reported in the study: one case of FHR abnormality in the mifepristone group, and two cases of acute fetal distress - one in each group. There were no deaths in the study.

	Treatment group		
	Mifepristone	Placebo	P
24 hours	3.18	3.23	0.951
	(1.96 - 4.41)	(2.02 - 4.44)	
48 hours	3.46	4.81	0.267
	(1.94 - 4.98)	(2.88 - 6.74)	
72 hours	3.63	5.23	0.206
	(2.08 - 5.19)	(3.22 - 7.25)	

Table 8.5. Maternal assessment of fetal movement - mean time (95% CI) to fetus kicking 10 times, measured in hours.

Neonatal safety

The frequency of neonatal adverse event reporting was also similar for the two groups. The percentage of neonates with a one minute Apgar score of 6 was 40% and 30% in the mifepristone and placebo groups respectively. Immediate resuscitation with intermittent positive pressure ventilation was required by 12% and 10% of neonates in the mifepristone and placebo groups respectively. There were no neonatal deaths in the study.

The normal range for blood glucose was defined as 2.2 to 4.4 mmol/L. Glucose concentrations transiently below 2.2 mmol/L were accepted without further investigation. However, persistently or markedly low glucose levels were investigated

with a glucose oxidase test at the discretion of the neonatal paediatricians. As shown in table 8.6 three hour Dextrostix results were significantly lower in the mifepristone group compared with placebo ($p< 0.05$). Two cases of hypoglycaemia were reported as serious adverse events - one in each treatment group (mifepristone - 1.0 mmol/L, placebo - 0.6 mmol/L). In both instances glucose levels were within the normal range at the 12 hour assessment.

There were no significant differences in cord pH, ACTH and cortisol results (Table 8.7). The laboratory reference range for ACTH was <65mU/L, and the reference range for cortisol was 0 - 695 mmol/L. The limit of detection for the mifepristone assay was <0.01 mg/L. There were no significant abnormalities on biochemical or haemoatological screening in either group.

	Treatment group		
	Mifepristone 50 mg	Placebo	P
Glucose:			
1 hour	3.0 (2.6 - 3.4)	3.2 (2.9 - 3.6)	0.331
3 hour	2.6 (2.3 - 2.9)	3.1 (2.7 - 3.5)	0.031
12 hour	3.1 (2.8 - 3.3)	2.9 (2.7 - 3.1)	0.327

Table 8.6. Neonatal glucose concentrations by treatment group. Analysed by ANOVA. Values given as mean (95% CI).

	Treatment group		
	Mifepristone 50 mg	Placebo	P
pH	7.26 (7.21 - 7.31)	7.26 (7.19 - 7.33)	0.98
ACTH (mU/L)	41.8 (21.8 - 61.7)	58.8 (11.5 - 106.2)	0.483
Cortisol (mmol/L)	498.6 (426.3 - 570.9)	467.3 (362.0 - 573.0)	0.604
Mifepristone (mg/L)	0.02 (0.01 - 0.04)	0.005 (0.004 - 0.005)	0.021

Table 8.7. Biochemical parameters by treatment group. Analysed by ANOVA.
Values given as mean (95% CI).

Discussion.

Ideally the aim of induction of labour should be to mimic the physiological process as closely as possible. The onset of labour is not abrupt, but rather the end point of a gradual change in uterine contractility, as elegantly demonstrated by Caldeyro-Barcia (1958). In this work he defined the phase of prelabour which is a prerequisite of successful parturition. Not only are important changes occurring with regard to uterine contractility during this time, but in addition, and independent from this, biochemical changes are taking place within the cervix to promote cervical ripening. The importance of cervical ripening is highlighted by the crucial role the state of the cervix plays in determining the success of labour induction. Women with an unfavourable cervix, that is those who have not gone through the stages of prelabour, present the greatest challenge with regard to labour induction where acceleration of the normal physiological changes is required.

The key to successful induction of labour is therefore to replicate pharmacologically the process of physiological cervical ripening. The most effective cervical ripening agents currently employed are the prostaglandins (Keirse, 1993). However, they do not have a selective effect on the cervix, but may promote ripening at the expense of stimulating uterine contractility, which in the face of an unripe cervix will be inefficient and stressful for the fetus and mother.

As discussed in chapter one, a number of biochemical changes take place within the cervix with advancing gestation of pregnancy. These include increased tissue hydration, a reduction in collagen concentration, altered composition of the extracellular matrix and infiltration of inflammatory mediators. Although there is no direct evidence to link progesterone with the timing of labour onset in humans, there is a considerable wealth of indirect evidence from investigation into the effects of various

progesterone antagonists to support a major role for this hormone in the control of parturition.

The mechanism of action of antiprogestins in promoting cervical ripening has been extensively investigated in a number of animal models. In pregnant rats treatment with three different antiprogestins, onapristone, lilopristone and RU 486, resulted in a dose-dependent increase in cervical softening 15 hours post treatment, and importantly these changes occurred long before the onset of labour suggesting that cervical changes precede labour after antiprogestin treatment (Chwalisz et al, 1991). Similarly, dramatic softening and dilatation of the cervix occurs in onapristone-treated guinea pigs prior to the onset of labour (Chwalisz et al, 1991). Histological examination of guinea pig cervices treated with onapristone demonstrates that these changes are associated with dissolution and disaggregation of collagen fibrils and increased hydration of the inter-fibrillar spaces (Hegele-Hartung et al, 1989). In addition there was an inflammatory cell infiltration of polymorphonuclear granulocytes, mast cells and active fibroblasts. In these experiments similar findings were seen in control animals just before term, that is animals undergoing physiological ripening. The mechanism of antiprogestin-induced ripening therefore appears to mirror the physiological process. These authors also found that there was no difference in the number of gap junctions between smooth muscle cells in the cervix in the treated and control groups, in contrast to the effect of antiprogestin treatment on myometrial cells which induces a massive increase in gap junction formation (Garfield et al, 1987).

The efficacy of RU 486 for induction of labour at term was first demonstrated by Frydman et al (1992). In this double-blind, placebo-controlled trial 120 women with an unfavourable cervix (modified Bishop score ≤ 4) were randomised to receive 200 mg RU 486 daily for two consecutive days or placebo. Induction of labour was planned for day four, and PGE_2 or amniotomy and oxytocin were employed. 54% of

women in the RU 486 group established in spontaneous labour without further intervention compared with 18% in the placebo group ($p<0.001$). We were not able to confirm this finding in the present study which is perhaps not surprising given the much smaller dose in the active treatment group (50 mg versus 400 mg in total). Of those women who had not gone into labour in Frydman's study (1992) significantly fewer in the mifepristone group had an unfavourable cervix on day four compared with placebo. In our study partial success was defined as the onset of spontaneous labour within the treatment period with the intervention of amniotomy or syntocinon infusion (or both), or a modified Bishop score ≥ 6 at the 72 hour assessment. Although failing to reach significance ($p=0.059$) there were more partial successes in the mifepristone group. This finding does suggest that mifepristone may be having some preparatory effect on the cervix over this time period even in a dose of 50 mg.

In view of the fact that the 50 mg dose of mifepristone appears to be too low to promote any major effect with regard to cervical ripening and induction of labour, it is not surprising that there was no significant difference in the labour characteristics of the treatment groups. There was a significant difference in the caesarean section rate for the two groups: 8% and 32% for the mifepristone and placebo groups respectively. The indication for caesarean section in the placebo group was arrest of labour in 50% of the cases. One attractive theory to explain the lower section rate in the mifepristone group is that the uterus has been "primed" and is therefore more responsive to exogenous oxytocic agents as a result of treatment. However, numbers are small and it is difficult to draw firm conclusions from this finding, particularly given the similar labour characteristics of the two groups.

Transplacental passage of mifepristone has been established. Frydman et al (1985) demonstrated that mifepristone crosses the placenta in the second trimester and that the concentration of mifepristone in fetal cord blood increases exponentially with time.

The lowest concentration (20 ng/mL) was found 30 minutes after administration, with the highest concentration (400 ng/mL) occurring 18 hours after oral administration. The authors suggested that these findings indicate an active transport mechanism, with fetal concentrations on average one third of maternal levels. Similar findings have been demonstrated in nonhuman primates (Wolf et al, 1988) where the maternal: fetal ratio of mifepristone fell from 3:1 in the second trimester to 6:1 in the third trimester. The authors concluded that the efficacy of placental transport may decrease with advancing gestation and that the theoretical risks of fetal side effects could be reduced in the third trimester.

The importance of transplacental passage lies in the fact that in addition to its antiprogestagenic action, mifepristone is known to possess antiglucocorticoid activity (Spitz and Bardin, 1993). In nonhuman primates the administration of mifepristone results in potent inhibition of prolactin secretion and marked elevation of circulating ACTH and cortisol concentrations (Healy et al, 1983). The increase in ACTH and cortisol is dose-dependent, with peak ACTH elevation occurring 1-2 hours post administration, and peak cortisol concentrations occurring 3-4 hours after mifepristone treatment. Similarly, in humans mifepristone will induce a sustained elevation of cortisol and lipotropin, which behaves in parallel with ACTH (Bertagna et al, 1984). Because of the antagonism of glucocorticoid action at target tissues there is the potential for hypotension and hypoglycaemia in the neonatal period.

In this study hypoglycaemia was reported as a serious adverse event in two infants; one in each treatment group. In both cases the blood glucose concentrations had returned to within the normal range by the 12 hour assessment. There was no significant difference in blood glucose monitoring in the neonatal period between the groups apart from the three hour assessment at which glucose concentrations were significantly lower in the mifepristone group compared with placebo. However, the

concentrations at this time point were still within the normal range. These findings are therefore reassuring with regard to the theoretical risk of problems with glycaemic control in the newborn period following mifepristone therapy, and are in accord with the rate of transient hypoglycaemia identified in Frydman's trial. Also reassuring is the finding that there was no significant difference in ACTH and cortisol concentrations in samples from cord blood at delivery, and that these results were within the normal range.

This study has demonstrated that a 50 mg dose of mifepristone is insufficient to promote cervical ripening and induction of labour in women with an unfavourable cervix. However, some preparatory changes appear to be taking place within the cervix in response to treatment suggesting that although a higher dose is required, this may not need to be as high as the total dose employed in Frydman's trial. We have also been able to confirm that mifepristone is not associated with any adverse events in the neonatal period. In particular there were no complications resulting from its antiglucocorticoid effect. The study concentrated on blood glucose monitoring and hypoglycaemia, but although blood pressure was not formally assessed there were no adverse events reported relating to problems with blood pressure control. Similarly, there were no differences in ACTH and cortisol measurements, although it is acknowledged that levels obtained at delivery do not give a direct indication of the response of the fetal pituitary-adrenal-axis in utero, particularly in the hours immediately following treatment. However, there was no difference in fetal monitoring tests such as cardiotocography and assessment of fetal movement over the time period for the study which is again a reassuring observation.

Mifepristone is clearly an effective cervical ripening agent but further dose-finding studies are required to establish the optimal dose for this clinical indication at term. Certainly it has been demonstrated that a variety of doses are equally effective in

facilitating termination of pregnancy in the first trimester (Baird, 1993), and obviously the risk of side effects for both mother and fetus is reduced the smaller the dose employed. Not only is further investigation into the use of antigestagens at term required in order to widen our options for achieving pharmacological ripening of the cervix, but also to advance our understanding of the mechanisms involved in the control of parturition which in turn has important implications for the management and prevention of preterm labour.

Conclusions

The inappropriate timing of parturition confers a number of significant sequelae. Preterm labour is associated with considerable perinatal morbidity and mortality, and represents the major cause of death in structurally and karyotypically normal babies. It is, therefore, a cause of considerable emotional distress, and has important implications for health service resources. Alternatively, clinical situations can arise which indicate interruption of pregnancy, namely induction of labour, necessitating pharmacological intervention. Labour induction is associated with increased morbidity for both mother and baby, and it is recognised that success is more likely if physiological mechanisms are replicated. In order to make any impact on these clinical problems, further knowledge regarding the mechanisms that control parturition is required.

Prostaglandins are thought to play a major role in the process of labour, although the precise stimulus for their production has yet to be established. The fetal membranes, comprising amnion and chorion, and decidua are an important site of prostaglandin synthesis and metabolism (Okazaki et al, 1981a). The increase in prostaglandin concentrations seen in association with labour could therefore be the result of increased prostaglandin synthesis, a reduction in prostaglandin metabolism, or a combination of the two within these tissues. Previous work has investigated changes in each tissue in isolation. Clearly it is important to look at potential changes in synthesis and metabolism in parallel. Such parallel experiments investigating amnion and chorion demonstrated that prostaglandin synthesis by amnion is increased in spontaneous labour compared with elective caesarean section, but that there is no alteration in prostaglandin metabolism between the two groups (chapter four). These findings are in accord with Okazaki et al (1981a) and Cheung and Challis (1989), the former investigating amnion and the latter investigating chorion.

The increase in prostaglandin synthesis by spontaneous labour amnion may reflect increased COX activity within this tissue. Phorbol esters, via their action on protein kinase C, can stimulate prostaglandin production by increasing substrate availability and COX activity. Spontaneous labour and elective section tissue produced significantly more prostaglandin E_2 in response to treatment with phorbol ester compared with baseline production, but the significantly higher baseline production by spontaneous labour amnion compared with elective section amnion was not maintained. These findings suggest that substrate availability, lower concentrations of COX, or a combination of both, are limiting factors in prostaglandin production by elective section tissue. The stimulatory effect of phorbol ester on elective section tissue may be due to an increase in inducible COX (COX-2) activity, which in spontaneous labour tissue may already be maximally stimulated, hence the lack of difference in prostaglandin production by the two groups of tissue following this treatment.

The importance of COX-2 activity in the generation of prostaglandins is highlighted by a number of studies. Using reverse transcriptase polymerase chain reaction technology Slater et al (1994) have demonstrated an approximate 100-fold increase in COX-2 mRNA compared with COX-1 mRNA in human amnion at term. An increase in COX-2 expression, with no change in COX-1 expression, in assocation with labour has been confirmed by further investigations (Slater et al, 1995; Hirst et al, 1995). In addition, it has been demonstrated that factors capable of inducing COX such as PMA, IL-1β and EGF do so at the level of COX-2 (Trautman et al, 1996).

There was no difference in the metabolism of exogenous prostaglandins by chorion tissue in association with labour, which implies that there is no change in PGDH activity, and is in keeping with the findings of Cheung et al (1990) regarding placental PGDH localisation in labouring and non-labouring tissue. However, this may not

accurately reflect the *in vivo* situation since there is evidence to suggest regional differences in PGDH activity within the uterus. Van Meir et al (1997a) have demonstrated that PGDH activity was highest in chorion from the region of the internal os in patients not in labour, but was significantly lower in this area compared with other regions of the fetal membranes in association with labour. This loss of PGDH activity, and hence reduced prostaglandin metabolism, near the internal os would facilitate prostaglandin transfer to the myometrium and cervix to promote contractility and ripening respectively. There is also evidence to support a reduction in PGDH activity in fetal membranes in association with some cases of preterm labour (Van Meir et al, 1997b).

Progesterone and dexamethasone were without effect on prostaglandin metabolism (chapter four). Treatment with the progesterone antagonist mifepristone (RU 486) resulted in a significant reduction in the metabolism of exogenous PGE_2 in spontaneous labour tissue, and was without effect in elective section tissue. This suggests that PGDH activity is reduced by treatment with mifepristone, which agrees with the *in vivo* decidual studies of Cheng et al (1993). The finding that mifepristone had no effect on elective section tissue implies that an endogenous factor specific to labour is integral to this process. Chorion expresses 3β-hydroxysteroid dehydrogenase (3β HSD) which converts pregnenolone to progesterone. Since progesterone may maintain PGDH expression it would be interesting to determine whether there is a localised reduction in progesterone concentrations, for example due to decreased 3β HSD activity, occurring in parallel with reduced PGDH expression.

The concept that the fetus has a role in the timing of parturition is an attractive one, with labour occurring once the fetus is appropriately mature. However, whilst there is evidence to suggest a role for the fetus and placental unit in the timing of labour onset, (section 1.8.2), no direct evidence for fetal control is as yet available in human

parturition, in contrast to the ovine model. One route by which the fetus might potentially control the onset of labour is via the amniotic fluid. A substance(s) in amniotic fluid could stimulate prostaglandin production by amnion, with which it is in direct contact. Alternatively, or additionally, transfer across the amnion could occur of a factor which can inhibit prostaglandin metabolism by chorion, the net result being an increase in prostaglandin concentrations. The effect of amniotic fluid on prostaglandin production by amnion has been investigated (Dowling et al, 1991; Cohen et al, 1991), but no work has studied the effect of amniotic fluid on metabolism by chorion, or investigated both tissues in parallel.

Spontaneous labour and elective caesarean section amniotic fluid stimulated prostaglandin production by amnion (chapter five). The stimulatory effect was significantly greater for spontaneous labour fluid, suggesting that there is a factor present in labouring fluid which is either absent from non-labouring fluid, or present in lower (stimulatory factor) or higher (inhibitory factor) concentrations compared with labouring fluid. Amniotic fluid had no effect on the metabolism of exogenous prostaglandins by chorion (chapter five) demonstrating that any increase in prostaglandin concentrations occurring in response to a substance in amniotic fluid results from an increase in prostaglandin synthesis and not a reduction in prostaglandin metabolism.

Potential regulatory mechanisms involved in the stimulatory effect of amniotic fluid were investigated in chapter six. These experiments demonstrate that new protein synthesis and protein kinase C are involved in basal production of prostaglandins by amnion and chorion. The stimulatory effect of amniotic fluid on prostaglandin synthesis is also mediated, at least in part, by protein kinase C activation and new protein synthesis. The inhibition of amniotic fluid's stimulatory effect on prostaglandin synthesis by cycloheximide may reflect inhibition of COX production /

activity. As discussed earlier the role of protein kinase C may be to increase substrate availability for prostaglandin production, and / or stimulate COX activity. The stimulatory effect of amniotic fluid was not completely abolished by cycloheximide or staurosporine suggesting that additional mechanisms may play a part. The experiments also demonstrated that tyrosine kinase is involved in the stimulatory effect of amniotic fluid lending further support to the importance of signal transduction mechanisms in this process.

No attempt has been made to characterise the substance(s) present in amniotic fluid which is responsible for the stimulatory effect on prostaglandin production. Cytokines, which are present in increased concentrations in amniotic fluid of labouring compared with non-labouring women at term, are possible candidates. They are capable of stimulating prostaglandin production by amnion and chorion, and it has been demonstrated that the mechanisms whereby this is achieved involve protein kinase C and COX-2. The fetal membranes produce a number of cytokines which, as discussed previously, are able to induce expression of each other. Whether the cytokines present in amniotic fluid are the result of a signal from the fetus, or whether their original source is the chorion and amnion, produced as a result of a trigger independent of the fetus remains unknown. However, there is potential for a complex cascade of events at the feto-maternal interface which could be crucial to the timing of parturition.

The process of cervical ripening is central to the appropriate timing of labour. The cervix is known to ripen during the phase of prelabour, described by Caldeyro-Barcia (1958), during which time it changes from a rigid sphincter responsible for maintaining the uterine contents, to a soft compliant structure which enables easy passage of the fetus during labour. These important changes in the cervix occur in the weeks prior to the onset of labour. This suggests that the trigger for labour onset is

not simply an acute event resulting in immediate regular uterine contractility, but rather that there are regulatory mechanisms that allow a gradual build up to active labour. What these mechanisms are in humans remain to be accurately defined, as does the switch from prelabour to labour.

In order to replicate physiological ripening, pharmacological agents should promote cervical ripening without having a significant effect on uterine contractility, thus reducing the maternal and fetal morbidity associated with labour induction in the presence of an unripe cervix. Relaxin is a hormone that has the potential to achieve this effect since it facilitates connective tissue remodelling, and has an inhibitory effect on myometrial contractility in some species. However, the exact role, if any, of relaxin in human parturition remains unclear. Chapter seven presents a randomised, double-blind, placebo-controlled trial investigating the efficacy of recombinant human relaxin as a cervical ripening agent, which because of its structure may produce superior results to those obtained in earlier trials employing porcine relaxin (MacLennan et al, 1980; Evans et al, 1983). The trial specifically investigated its use in women with an unfavourable cervix (modified Bishop score ≤ 4) since this group presents the greatest challenge with regard to labour induction. This trial demonstrated no effect of human recombinant relaxin on cervical ripening at the three doses tested (1, 2 and 4 mg). This could be due to inappropriate dosage or route of administration, or may simply reflect the fact that relaxin is not a significant hormone in human parturition. The success of the earlier studies with porcine relaxin may be explained by an immunological reaction leading to inflammatory cell infiltration of the cervix and collagenase release, which would be in keeping with the large body of evidence suggesting that physiological ripening is an inflammatory-mediated process (Liggins et al, 1981).

There is an infiltration of neutrophils and macrophages into the human cervix at term (Junqueira et al, 1980; Osmers et al, 1992), and since these cells are a source of collagenases (Ito et al, 1987) they may provide an important link in the ripening process. Similar findings have been demonstrated in animal models (Hegele-Hartung et al, 1989), and interestingly this infiltration appears to be under the control of progesterone, since administration of progesterone antagonists increases the inflammatory response (Chwalisz et al, 1991). There is evidence demonstrating that the effect of progesterone withdrawal is cytokine mediated, with progesterone inhibiting the production of cytokines in a number of tissues (Kelly et al, 1992; Kelly et al, 1994). In addition pharmacological ripening with topical cytokines in the animal model results in histological changes within the cervix that are similar to those induced by antiprogestational agents (Chwalisz et al, 1994).

It has been suggested that progesterone withdrawal, either physiological or pharmacological, may activate the cytokine cascade and hence cervical ripening (Chwalisz et al, 1994) and the above findings go some way to explain the efficacy of mifepristone for interruption of pregnancy at all gestations. In addition to its effect on cervical ripening mifepristone increases myometrial responsiveness to endogenous and exogenous prostaglandins and therefore has a dual effect of preparing both myometrium and cervix for labour. Additional prostaglandin therapy will increase the number of women delivering following mifepristone therapy at all gestations and this would seem an appropriate approach therefore to induction of labour at term. In view of the more physiological approach that mifepristone may provide for cervical ripening it has been investigated for this indication in human pregnancy at term (Frydman et al, 1992), when it was demonstrated that a dose of 400 mg promoted spontaneous labour in significantly more women compared with placebo.

Chapter eight presents the results of the first part of a dose-finding placebo-controlled trial with the aim of establishing the lowest dose of mifepristone necessary for cervical ripening and labour induction in women with an unfavourable cervix at term. The results demonstrate that a dose of 50 mg is not sufficient to promote cervical ripening and labour onset without further intervention. However, the number of women labouring successfully with further intervention (eg. amniotomy +/- syntocinon) was higher in the mifepristone group suggesting that mifepristone may be having some preparatory effect on the cervix. Thus a slightly higher dose may produce a significant effect, and in fact in the second stage of this dose-finding study, which did not form part of the work of this thesis, a 200 mg dose of mifepristone was significantly more effective than placebo in promoting spontaneous labour and an improvement in Bishop score when analysed in combination (Elliott et al, 1998). Importantly, there was no difference in monitoring of fetal and neonatal safety between the treatment and placebo groups.

Mifepristone is an attractive cervical ripening agent because it promotes histological changes, at least in animal models, within the cervix that are similar to physiological ripening. In addition, there is evidence for an effect on intrauterine tissues, as discussed earlier. There is a reduction in PGDH immunoreactivity in decidua from women treated with mifepristone in early pregnancy (Cheng et al, 1993), and a reduction in the metabolism of exogenous prostaglandins by chorion explants collected from spontaneous labour at term treated with mifepristone *in vitro* (chapter 3). Both of these effects would lead to an increase in prostaglandin concentrations within the uterus with the potential for stimulating myometrial contractility. Thus progesterone withdrawal, induced by mifepristone, may have an action on myometrial gap junction formation, prostaglandin production within fetal membranes and decidua, and cervical ripening. There is no direct evidence to support the theory of progesterone withdrawal in human parturition. However, it has been suggested that events may occur within the

intrauterine tissues to effect local progesterone withdrawal which would not be reflected in peripheral progesterone concentrations. These changes could then facilitate mechanisms such as cytokine infiltration leading to cervical ripening and important interactions at the feto-maternal interface resulting in the production of myometrial stimulants, namely prostaglandins.

Our understanding of the mechanisms governing the onset of parturition in humans is fragmentary. There is at present no direct evidence indicating that the process is under the control of the fetus, which would initiate labour when appropriately mature, although this is likely at least in part. It may be that the intrauterine tissues, the feto-placental-membrane unit, including amnion and chorion are of major importance in determining events leading to the onset of labour. Prostaglandins are thought to be central to the process of labour and the work presented in this thesis demonstrates that prostaglandin synthesis by fetal membranes is increased in association with spontaneous labour, while prostaglandin metabolism by chorion is unaffected by this process.

Amniotic fluid is capable of stimulating prostaglandin synthesis by amnion, and this is significantly greater in response to spontaneous labour fluid, perhaps indirectly indicating a role for the fetus in the increased production of prostaglandins. Amniotic fluid has no effect on the metabolism of prostaglandins by chorion lending further support to the hypothesis that the increase in prostaglandin concentrations seen in association with labour is a result of increased synthesis and not a reduction in metabolism. Mechanisms by which amniotic fluid stimulates prostaglandin production include activation of protein kinase C, tyrosine kinase and new protein synthesis. Candidates for these effects include members of the cytokine family such as the interleukins -1, 6 and 8 which are known to be present in amniotic fluid in increased concentrations in association with labour at term, and which are capable of stimulating

prostaglandin production by human fetal membranes. This leads to the concept that labour may also be the result of an inflammatory reaction, as has been suggested for cervical ripening. A combination of hormonal changes are likely to prime the uterus and cervix for this proinflammatory process in turn mediated by candidates such as cytokines and prostaglandins which promote ripening and uterine contractility. The preparatory changes within the cervix and uterus which culminate in spontaneous labour occur in concert and are likely to be orchestrated by the feto-placental-membrane unit.

The work described in this thesis raises a number of areas for continuing research. It would be important to investigate the effect of *in vivo* treatment with mifepristone at term on prostaglandin synthesis and metabolism by amnion and chorion, and to establish any modification in the response to inflammatory mediators. In view of the dramatic inhibition of amniotic fluid-stimulated prostaglandin production following treatment with staurosporine the role of protein kinase C in parturition merits further investigation. In addition, phospholipase inhibitors could be employed to determine the role of phospholipase A_2 in amniotic fluid-stimulated prostaglandin production, and the role of COX could be more accurately assessed using specific COX-2 inhibitors. It would also be interesting to explore the hypothesis that there may be regional variations in prostaglandin synthesis and metabolism depending on the site from which the membranes are sampled. Further clinical trials are required to determine the correct dose of mifepristone for induction of labour at term and to establish the safety of this agent. The optimal combination of mifepristone and PGE_2 requires investigation and consideration might be given to the potential of the PGE_1 analogue misoprostil in combination with mifepristone. Another approach to cervical ripening might be topical application of cytokines which could have a local effect on the cervix and perhaps influence prostaglandin production by the fetal membranes adjacent to the cervix promoting a cascade resulting in uterine contractility.

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Appendix One. Abbreviations

The following abbreviations have been employed throughout.

AA	Arachidonic Acid
ANOVA	Analysis of variance
CI	Confidence interval
COX	Cyclooxygenase
DAG	Diacylglycerol
EGF	Epidermal growth factor
GAG	Glycosaminoglycan
HPA	Hypothalamo-pituitary-adrenal
IL	Interleukin
MOX	Methyloxime
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
PAF	Platelet activating factor
PG	Prostaglandin
PGDH	Prostaglandin dehydrogenase
PGE_2	Prostaglandin E_2
PGEM	Prostaglandin E metabolite
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFM	Prostaglandin F metabolite
PGHS	Prostaglandin endoperoxide synthase
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PMA	Phorbol myristoyl acetate
SD	Standard deviation

SEM	Standard error of the mean
TPA	Teradecanoyl phorbol acetate
TNF	Tumor necrosis factor

Appendix Two.

Copies of Papers Arising From This Thesis

© European Journal of Endocrinology 1995, 133: 527–33

Changes in prostaglandin synthesis and metabolism associated with labour, and the influence of dexamethasone, RU 486 and progesterone

Janet E Brennand[1], Rosemary Leask[2], Rodney W Kelly[3], Ian A Greer[1] and Andrew A Calder[2]

Department of Obstetrics and Gynaecology[1], University of Glasgow; Department of Obstetrics and Gynaecology[2], University of Edinburgh; MRC Unit[3], Centre for Reproductive Biology, Edinburgh, UK

Brennand JE, Leask R, Kelly RW, Greer IA, Calder AA. Changes in prostaglandin synthesis and metabolism associated with labour, and the influence of dexamethasone, RU486 and progesterone. Eur J Endocrinol 1995;133:527–33. ISSN 0804–4643

The objective was to compare the changes in prostaglandin synthesis and metabolism occurring within the fetal membranes that are associated with the onset of parturition and to study the effect of steroid hormones on prostaglandin metabolism. A tissue explant study was made of discs of amnion and chorion obtained from 24 pregnant women at 37–42 weeks' gestation following spontaneous labour and delivery (12 women) and elective caesarean section (12 women). Significantly more prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ were synthesized by amnion obtained following spontaneous labour than elective caesarean section. Arachidonic acid stimulated both PGE_2 and $PGF_{2\alpha}$ synthesis by amnion in both groups. Phorbol myristoyl acetate stimulated PGE_2 synthesis in both groups. There was no difference between the groups in the capacity of the chorion to metabolize prostaglandins. Mifepristone (RU 486) reduced the metabolism of added PGE_2 following spontaneous labour, while dexamethasone and progesterone had no effect on prostaglandin metabolism. In conclusion, the increase in concentration of PGE_2 and $PGF_{2\alpha}$ associated with the onset of spontaneous labour is the result of an increase in synthesis rather than a reduction in metabolism. There was no decrease in metabolism to account for the increase in prostaglandin concentrations and, with the exception of mifepristone, metabolism was not altered by the addition of steroid hormones.

Janet Brennand, Department of Obstetrics and Gynaecology, Glasgow Royal Maternity Hospital, Rottenrow, Glasgow G4 0NA, UK

Prostaglandins are intimately involved in the process of parturition. This is evidenced by the increase in concentrations of PGE_2, $PFG_{2\alpha}$ and their metabolites in maternal plasma (1–3), urine and amniotic fluid (3, 4) at the onset of spontaneous labour. The fetal membranes appear to be the source of the increase in prostaglandins seen with labour. The amnion produces PGE_2 and has little metabolizing activity (5), whereas the chorion is the major site of prostaglandin metabolism, being rich in prostaglandin dehydrogenase (5–7). This enzyme converts PGE_2 and $PGF_{2\alpha}$ to their respective metabolites 13,14-dihydro-15-keto-PGE_2 (PGEM) and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM). There are two mechanisms by which this increase in prostaglandin levels can be effected: firstly, an increase in their synthesis, or secondly, a decrease in their metabolism.

It is well established that prostaglandin production by amnion is increased in spontaneous labour (6, 8, 9). However, in order to exert their uterotonic effect on the myometrium, prostaglandins synthesized by the amnion would have to escape the metabolic activity of prostaglandin dehydrogenase within the chorion, and there are conflicting reports as to whether or not this is possible. Some reports have confirmed that transfer of prostaglandins across full-thickness membranes does occur, with the majority of the prostaglandin remaining intact (10, 11), whereas others have shown that transfer does not take place (12). Any reduction in the metabolizing capacity of the chorion could potentially enhance prostaglandin transfer. There is little information available on changes in prostaglandin metabolism associated with labour, with one report suggesting that there is no difference in the metabolizing capacity of pre- and post-labour tissues (6).

Although the effect of steroid hormones on prostaglandin production has been examined extensively (13, 14), this is not the case for prostaglandin metabolism and no previous study has examined prostaglandin metabolism in parallel. It has been shown that antiprogestagenic steroids alter prostaglandin metabolism in the guinea-pig (15), but there is little information on the effect of progesterone and corticosteroids on prostaglandin metabolism in chorion.

The aim of this study was to assess, in parallel, the alterations in prostaglandin synthesis and metabolism

in amnion and chorion, respectively, that are associated with spontaneous labour and to investigate the effect of steroid hormones on prostaglandin metabolism.

Subjects and methods

Fetal membranes were obtained from women at term with uncomplicated pregnancies. Group one laboured spontaneously and achieved a vaginal delivery without oxytocin augmentation (N = 12), and group two underwent elective caesarean section for either breech presentation or previous caesarean section (N = 12).

The membranes were trimmed from the placenta immediately following delivery and transported in Dulbecco's phosphate-buffered saline solution (DPBS) containing heparin (10 U/ml). The amnion and chorion were separated manually and the decidua peeled from the chorion with fine-tissue forceps. The amnion and chorion were soaked in DPBS containing 40 mg/500 ml gentamicin (Sigma, UK) and amphotericin B 2.5 mg/500 ml (Sigma, UK) for 1 h. Previous work in the laboratory has shown that neither heparin nor the antibiotics affect prostaglandin synthesis or metabolism. The tissues then were rinsed thoroughly with DPBS.

Amnion tissue culture

Discs of amnion, 12 mm in diameter, were cut with a cork borer and each suspended on a 1-cm^2 piece of sterile capillary matting. Each disc then was submerged in a standard culture plate well containing 900 μl of complete culture medium. Complete culture medium consisted of RPMI 1640 (Gibco, UK) with 25 mmol/l HEPES buffer plus L-glutamine with the following additions: 50 ml/500 ml fetal calf serum (Gibco, UK); 5 ml/500 ml Penstrep solution (5000 IU/ml penicillin and 5000 mg/ml streptomycin; Gibco, UK); 5 ml of insulin–transferrin–sodium selenite media supplement (Sigma, UK).

The explant discs of amnion were treated with the following (final concentrations): 100 μmol/l arachidonic acid (Sigma, UK); 100 nmol/l phorbol myristoyl acetate (PMA) (Sigma, UK); 2.8 μmol/l indomethacin (Sigma, UK) or complete culture medium, the latter to act as control. The solutions of arachidonic acid, PMA and indomethacin were made up in complete culture medium. The volume added was 100 μl and the discs therefore were cultured in a total fluid volume of 1 ml. All experiments (N = 12 in each group) were performed in duplicate (i.e. two discs per experiment per patient, giving eight discs in total per patient).

The plates were incubated for 18 h in humidified 5% CO_2 in air at 37°C. Following incubation, 0.5 ml of incubation medium was aspirated from each well and oximated with an equal volume of methyl oximating solution. These samples were mixed thoroughly and stored at room temperature for 24 h and then at 5°C pending radioimmunoassay.

Chorion tissue culture

Chorion explant discs, 9 mm in diameter, were set up in culture medium as described for the amnion experiment and treated with one of the following (final concentrations): 1 μmol/l dexamethasone (Sigma, UK); 1 μmol/l progesterone (Sigma, UK); 1 μmol/l RU 486 (Roussel Laboratories) or complete culture medium, the latter to act as control. All steroid solutions were made up in complete culture medium. The explants were incubated for 18 h in humidified 5% CO_2 in air at 5°C.

The following solutions were added (final concentrations): 100 μmol/l arachidonic acid, 500 ng of PGE_2 (Upjohn Ltd, UK), 500 ng of $PGF_{2\alpha}$ (Upjohn Ltd, UK) and complete culture medium, the latter to act as control. Again, the solutions were made up in complete culture medium and the total volume of culture fluid was maintained at 1 ml. All experiments (N = 12 in each group) were performed in duplicate (i.e. two discs per experiment per patient, giving eight discs in total per patient). The explants were incubated for a further 4 h and then 0.5 ml of incubation medium was aspirated from each well for oximation, as described previously.

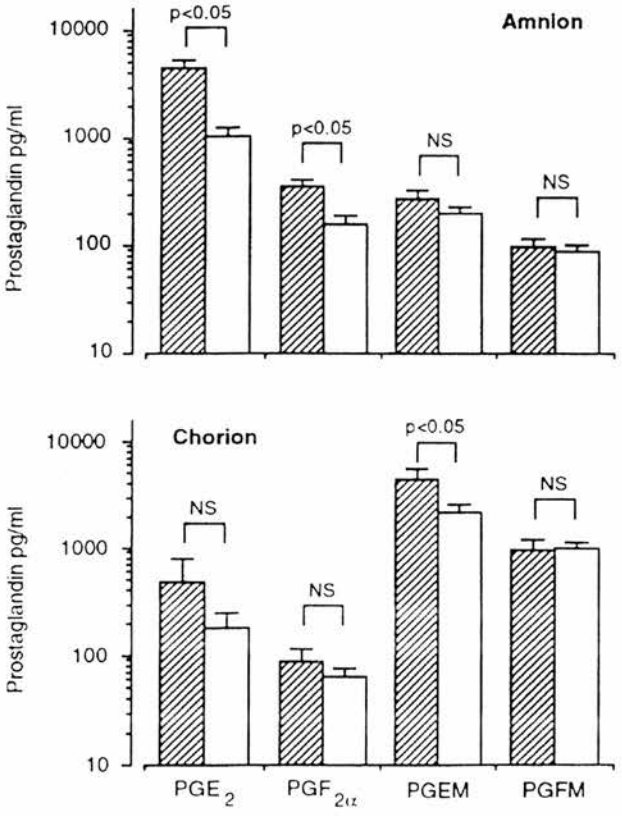

Fig. 1. Basal production of PGE_2, $PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGE_2 (PGEM) and 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) by amnion and chorion following spontaneous labour (▨) (N = 12) and elective caesarean section (□) (N = 12). Results expressed as means (SEM).

Radioimmunoassay

A competitive binding radioimmunoassay was used to determine the PGE_2, $PGF_{2\alpha}$, PGEM and PGFM content of the media. The technique has been described previously in detail (16, 17). In summary, up to 50 μl of the oximated culture medium was assayed. The labels employed are the iodinated (^{125}I) methyloximes of PGE_2, $PGF_{2\alpha}$, PGEM and PGFM (Pro-Gly-Tyr conjugate in each case). Antisera to the respective labels were raised in the rabbit. The assay was left overnight prior to separation of the antigen–antibody complex from the free antigen by a second antibody magnetic separation procedure. The intra-assay coefficient of variation was 12.3% for PGE_2, 10.5% for $PGF_{2\alpha}$, 8.4% for PGEM and 7.3% for PGFM. The interassay coefficients of variation were 13.3%, 13.9%, 13.9% and 13.3% for PGE_2, $PGF_{2\alpha}$, PGEM and PGFM, respectively. The sensitivity of the assay was 2 pg in all assays. This gives a limit of detection of approximately 40 pg per well.

Statistics

Analysis of variance (ANOVA) was used to analyse the data. Where the data were not distributed normally, log transformation was employed prior to ANOVA.

Results

Effect of labour on prostaglandin synthesis in amnion (Fig. 1)

Basal production of PGE_2 and $PGF_{2\alpha}$ was significantly higher in amnion obtained following spontaneous labour compared with elective caesarean section ($p < 0.05$). Neither PGEM nor PGFM production differed significantly between spontaneous labour and caesarean section. Production of $PGF_{2\alpha}$ was substantially less than that of PGE_2.

Effect of arachidonic acid, phorbol myristoyl acetate and indomethacin on prostaglandin synthesis in amnion (Fig. 2)

The addition of arachidonic acid resulted in significant stimulation of PGE_2 and $PGF_{2\alpha}$ synthesis by amnion obtained following both spontaneous labour and caesarean section in comparison with the basal synthesis

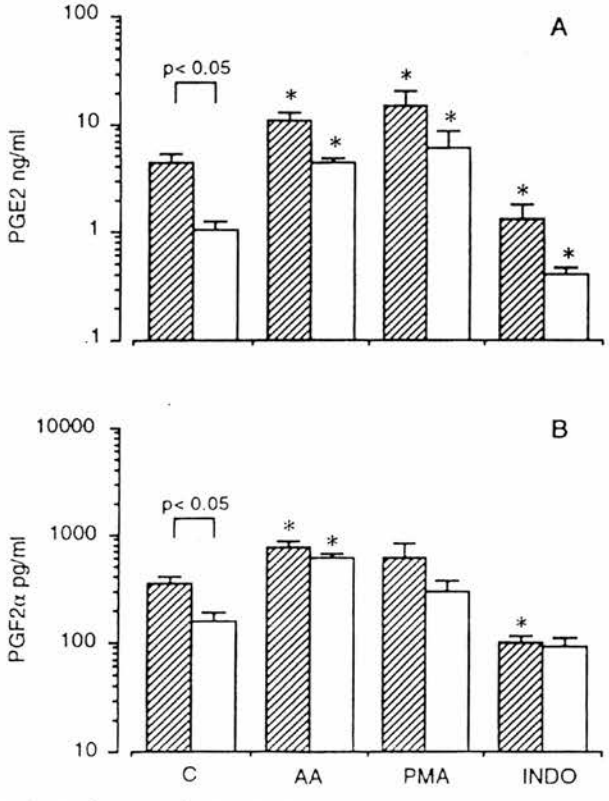

Fig. 2. Production of PGE_2 (ng/ml) (A) and $PGF_{2\alpha}$ (pg/ml) (B) by amnion following the addition of 100 μmol/l arachidonic acid (AA), 100 nmol/l phorbol myristoyl acetate (PMA) and 2.8 μmol/l indomethacin (INDO), compared with control (C): (▨) spontaneous labour (N = 12); (□) elective caesarean section (N = 12). Results expressed as means (SEM); *differs from respective control group, $p < 0.05$.

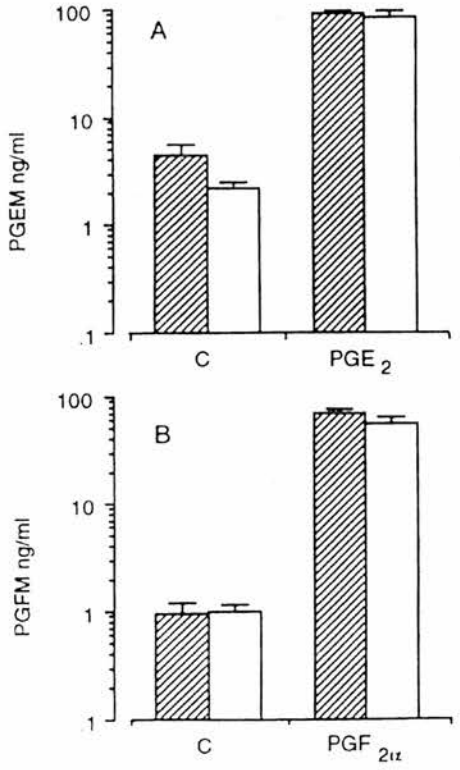

Fig. 3. Metabolism of added PGE_2 (500 ng) to 13,14-dihydro-15-keto-PGE_2 (PGEM) (A) and $PGF_{2\alpha}$ (500 ng) to 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) (B) by chorion following spontaneous labour (▨) (N = 12) and elective caesarean section (□) (N = 12). Results expressed as means (SEM).

in the two groups ($p < 0.05$) (Note: PGE_2 in ng/ml; $PGF_{2\alpha}$ in pg/ml). The percentage increase in prostaglandin production over basal levels was greater in the caesarean section tissue and this was significant for $PGF_{2\alpha}$ ($p < 0.01$). Phorbol myristoyl acetate stimulated PGE_2 production from amnion in both groups ($p < 0.05$). There was no significant stimulation of $PGF_{2\alpha}$ production following the addition of PMA in either group. Indomethacin inhibited PGE_2 production in both groups ($p < 0.05$). Also, $PGF_{2\alpha}$ production was inhibited by indomethacin in amnion obtained following spontaneous labour ($p < 0.05$).

Effect of labour on prostaglandin metabolism in chorion (Figs 1 and 3)

The major product of the chorion in both spontaneous labour and caesarean section groups was PGEM, and production was significantly higher in the spontaneous labour tissue ($p < 0.05$) (Fig. 1); PGFM was produced in smaller amounts and only minimal levels of PGE_2 and $PGF_{2\alpha}$ were recorded. There was no significant difference in the production of either primary prostaglandin or PGFM between the two groups. Metabolism of added PGE_2 to PGEM was the same in chorion from both groups (Fig. 3). Similarly, there was no difference in the metabolism of added $PGF_{2\alpha}$ to PGFM between the groups.

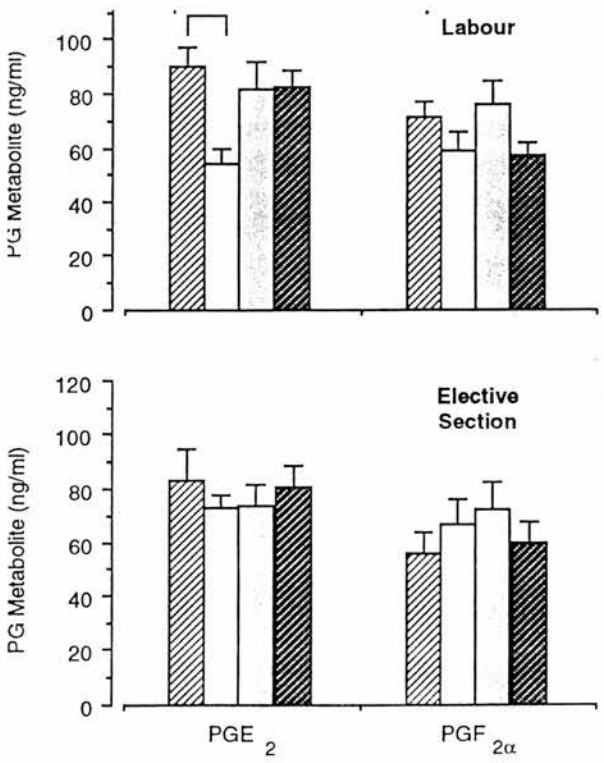

Fig. 4. Metabolism of added PGE_2 (500 ng) and $PGF_{2\alpha}$ (500 ng) to their respective metabolites by chorion following spontaneous labour ($N = 12$) and elective caesarean section ($N = 12$); control (▨) versus 1 μmol/l RU 486 (□), 1 μmol/l dexamethasone (■) and 1 μmol/l progesterone (▨). Results expressed as means (SEM).

Effect of steroids on prostaglandin metabolism in chorion (Fig. 4)

Dexamethasone and progesterone had no effect on prostaglandin metabolism. However, RU 486 decreased the metabolism of added PGE_2 in chorion obtained following spontaneous labour, but not in elective section tissue.

Discussion

The aim of this study was to compare changes in prostaglandin synthesis and metabolism in parallel by looking at both the amnion and chorion collected from each subject. The major product of the cultured amnion explants was PGE_2, which is in keeping with reports from others (5, 18). We were able to demonstrate a significant increase in PGE_2 production by amnion obtained following spontaneous labour, in keeping with the findings of previous studies (6, 8, 9). Although measured in much smaller concentrations, we also found a significant increase in the production of $PGF_{2\alpha}$ by the amnion following spontaneous labour, which could not be explained by any cross-reactivity because the PGE_2 cross-reactivity with $PGF_{2\alpha}$ assay was <0.02%. Okazaki et al. (5) were unable to demonstrate $PGF_{2\alpha}$ production by the amnion. However, Mitchell et al. (18) detected small amounts of $PGF_{2\alpha}$ in their superfusates of amnion tissue. The main source of $PGF_{2\alpha}$ is the decidua and any contribution that the amnion makes to increased $PGF_{2\alpha}$ concentrations is likely to be negligible. Indeed, the presence of $PGF_{2\alpha}$ in the culture system could be explained by non-enzymatic conversion from PGE_2, or by production by fibroblasts or macrophages present within the deeper layers of the amnion. Production of both PGEM and PGFM by the amnion was minimal, reflecting the virtual absence of prostaglandin dehydrogenase activity in this tissue (7).

In our experiment, the addition of arachidonic acid resulted in significant stimulation of PGE_2 and $PGF_{2\alpha}$ production by amnion obtained both before and after labour compared to basal concentrations. The mobilization of arachidonic acid from membrane glycerophospholipids by phospholipase A_2 is the rate-limiting step in prostaglandin production. An increase in the activity of this enzyme with advancing gestation, but not with the onset of labour, has been described (19). Following the addition of arachidonic acid there was no longer a significant difference in prostaglandin production between the two groups, which suggests that the availability of arachidonic acid is a limiting factor in prostaglandin production by the elective section tissue.

The difference in basal production of PGE_2 and $PGF_{2\alpha}$ between the groups could be explained by a difference in the activity of prostaglandin H synthase (cyclooxygenase) in pre- and post-labour amnion. Lopez Bernal et al. (20) have suggested that maturation of the activity of PGHS occurs toward term. Further results supporting this theory come from Olson et al. (21). Increased expression of the cycloxygenase gene has been demonstrated in human fetal membranes and placenta with the onset of labour (22). If there was increased cyclooxygenase activity in the spontaneous labour tissue we would expect the significant difference in prostaglandin production between the two groups to persist following the addition of arachidonic acid. Our findings suggest that, provided adequate substrate is available, both groups have similar capacities for prostaglandin production. However, the effect of exogenous arachidonic acid on cyclooxygenase activity is not known, and it is possible that the arachidonic acid may have induced and saturated the enzymes, thus abolishing any differences seen previously.

Addition of the phorbol ester PMA resulted in increased production of PGE_2 by amnion in both groups compared to control values. Phorbol esters act by stimulating the activity of the enzyme protein kinase C (PKC), a calcium- and lipid-dependent protein kinase that is involved in the regulation of many cellular functions. The presence of PKC has been identified in human amnion and decidua vera tissues (23) and it is involved in arachidonic acid release and the activation of PGHS (24). There are two main isoforms of PGHS (25–27): PGHS I is the constitutive calcium-dependent form of the enzyme, whereas PGHS II can be induced by a number of agents, including phorbol esters. This study of amnion explants confirmed the stimulatory effect of phorbol ester on PGE_2 production (24). There was no effect on $PGF_{2\alpha}$ production, but this may simply be related to a lower level of production in comparison to PGE_2. There was no significant difference in response to PMA between the spontaneous labour and caesarean section amnions. These results suggest that PGHS activity is comparable in both groups. One explanation for this is that PGHS II activity has been induced maximally by PMA and any original difference in PGHS activity has been lost.

Significant PGEM production occurred in the chorion, which is likely to reflect PGE_2 production because of the metabolizing capacity of the chorion for prostaglandins via 15-hydroxy-prostaglandin dehydrogenase (PGDH), the predominant enzyme in this tissue (5). Production of PGFM, reflecting $PGF_{2\alpha}$ production, was substantially less than PGE_2/PGEM. These results are in accordance with those of Okazaki et al. (5). We found no difference in the metabolism of added PGE_2 and $PGF_{2\alpha}$ to their respective metabolites, confirming the report of Cheung and Challis (6) who demonstrated no change in the metabolism of PGE_2 added to cell cultures of chorion obtained pre- and post-labour. This is compatible with the finding that PGDH localization in the placenta and membranes is not altered in association with labour (7). It therefore appears that the increase in prostaglandin concentrations associated with spontaneous labour is not the result of a decrease in metabolic activity within the chorion.

Mifepristone (RU 486) is a well-established medical abortifacient and more recently has been employed successfully as an agent for labour induction at term (28). In vitro studies exploring the effect of RU 486 on endometrial (29) and early decidual cells (30) have shown that prostaglandin synthesis is stimulated, and metabolism inhibited, by the antiprogestin. In addition, work in the guinea-pig has shown decreased prostaglandin metabolism in myometrium and chorion following in vivo treatment with RU 486 (15). In our experiment, the addition of RU 486 had no effect on basal prostaglandin metabolite production in either group. However, the metabolism of added PGE_2 to PGEM was reduced significantly in the spontaneous labour tissue following RU 486 treatment. These findings point to a reduction in the activity or amount of prostaglandin dehydrogenase in the tissue following treatment with RU 486. Recent work on decidua obtained from women pretreated with RU 486 in early pregnancy has shown, by both a direct enzymatic method and immunohistochemistry, that the activity of PGDH is reduced in this tissue compared with the control (31). It was surprising that the reduction in PGE_2 metabolism following treatment with RU 486 was confined to spontaneous labour tissue. Although basal levels were comparable, there may still be a lower degree of prostaglandin dehydrogenase activity in tissues obtained following spontaneous labour that may not be evident under basal conditions in the culture system. When RU 486 is added, an additive or synergistic effect may result, so explaining the results seen in the experiments on chorion taken from women in spontaneous labour. Such a lower degree of prostaglandin metabolism within the tissue with RU 486 may be in keeping with previous in vivo studies (31). The increase in prostaglandin concentrations at the level of the fetal membranes that would result from such inhibition of metabolism could contribute to the enhanced uterine activity seen both in spontaneous labour and following treatment with RU 486. Clearly an endogenous tissue factor associated with labour is required, because the effect was not seen with tissues treated with RU 486 taken from women not in labour. It is also possible that the difference between changes in metabolism seen following in vivo treatment compared with that following in vitro treatment with RU 486 may reflect an intermediate step in this process.

Dexamethasone has been shown to inhibit prostaglandin production from placental and amnion cell cultures (32), and it appeared that this steroid is acting at the level of PGHS activity. Alternatively, an increase in PGDH activity with an associated increase in

prostaglandin metabolism would result in a decrease in prostaglandin concentrations. An increase in the tissue activity of PGDH in rat lung and kidney following treatment with prednisolone has been reported (33). However, we were unable to demonstrate increased prostaglandin metabolism in response to dexamethasone in our chorion explants. Similarly, Gibb et al. (34) were unable to demonstrate an increase in prostaglandin metabolism to account for the reduction in PGE_2 output by their chorion cells incubated in the presence of dexamethasone.

In sheep, progesterone withdrawal is a prerequisite to the onset of parturition. In humans, there is no decrease in circulating systemic progesterone concentrations prior to the onset of spontaneous labour. However, systemic steroid concentrations may not reflect local changes in steroid environment within the fetal membranes that may control the process of parturition in a paracrine fashion. In our experiment the addition of progesterone had no effect on prostaglandin metabolism. This is in contrast with the inhibitory effect of RU 486. One possible explanation for this is that endogenous progesterone is exerting tonic control over prostaglandin metabolism, which could not therefore be altered by additional progesterone but could be overcome by the antiprogestin.

In summary, this study has shown that prostaglandin synthesis from fetal membranes is increased in association with parturition but that there is no alteration in prostaglandin metabolism by chorion in association with labour. However, metabolism in spontaneous labour tissue can be inhibited by the addition of RU 486.

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Received January 10th, 1995
Accepted June 6th, 1995

British Journal of Obstetrics and Gynaecology
July 1997, Vol. 104, pp. 775–780

Recombinant human relaxin as a cervical ripening agent

*Janet E. Brennand *Registrar*, *Andrew A. Calder *Professor*, †Craig R. Leitch *Senior Registrar*, ‡Ian A. Greer *Professor*, §Min Min Chou *Research Fellow*, §Ian Z. MacKenzie *Consultant*

*Departments of Obstetrics and Gynaecology at *University of Edinburgh, †St Mary's Hospital, Manchester, and ‡University of Glasgow; §Nuffield Department of Obstetrics and Gynaecology, University of Oxford*

Objective The aim of this study was to investigate the efficacy and safety of recombinant human relaxin (rhRIx) as a cervical ripening agent in women with an unfavourable cervix before induction of labour at term.

Design A multi-centre, double-blind, placebo-controlled trial performed in Edinburgh, Glasgow and Oxford. Women were treated with 0, 1, 2 or 4 mg of rhRIx in a gel vehicle administered intravaginally. Analysis of variance tests were performed on all continuous variables, and Cochran Mantel-Haenszel tests employed for all discrete variables.

Participants Ninety-six women at 37 to 42 weeks of gestation with a singleton pregnancy and a modified Bishop score of ≤ 4 were recruited.

Results There was no significant difference in the change in modified Bishop score between the four treatment groups. The lengths of the first and second stages of labour were similar in all 4 groups. PGE_2 and oxytocin requirements were similar in all groups, as was the mode of delivery. There was no evidence that relaxin was absorbed systemically when given in this way.

Conclusion Recombinant human relaxin 1 to 4 mg, administered as an intravaginal gel, has no effect as a cervical ripening agent before induction of labour at term.

INTRODUCTION

The success of labour induction is influenced by the state of the cervix; induction of labour in the presence of an unripe cervix is associated with an increase in both maternal and neonatal morbidity[1]. Cervical ripening occurs during the phase known as pre-labour. Its exact mechanism remains unclear but the process results in structural changes within the cervix making it more compliant. These include a decrease in collagen concentration, an increase in water content and an alteration in proteoglycan/glycosaminoglycan ratio within the tissue. It is this process that we are striving to mimic when ripening the cervix pharmacologically. The ideal cervical ripening agent should have a selective effect on the cervix without any additional effect on uterine contractility, and in theory the polypeptide hormone relaxin would meet these requirements.

Relaxin was first identified in 1926 by Hisaw[2] who demonstrated its capacity to promote separation of the pubic symphysis in guinea-pigs. Relaxin facilitates connective tissue remodelling, and in some animals also inhibits myometrial contractility. In most species, cervical ripening before the onset of labour is associated with an increase in serum relaxin concentrations. However, this is not the case in humans and the exact role of this hormone in human parturition remains uncertain. Early clinical trials[3,4] employing porcine relaxin for cervical ripening in human subjects reported conflicting results which probably reflected the impurity of the agent at that time. The subsequent purification of porcine relaxin[5] led to renewed interest in this hormone. A number of studies showed that porcine relaxin had some therapeutic benefit as a cervical ripening agent in women[6,7], despite the primary peptide structure of porcine relaxin having only about 50% homology with that of human relaxin. The development of recombinant human relaxin by Genentech Inc (San Francisco, California, USA) has provided an agent that could have superior cervical ripening properties in women and has stimulated further research in this field.

Phase I studies conducted by Genentech demonstrated that recombinant human relaxin was safe and not associated with serious adverse effects, and that there was no maternal development of antibody to rhRIx following treatment[8,9]. Phase II studies were therefore initiated in Australia and the UK. This paper reports the results of the UK double-blind, three-centre study investigating the effect of recombinant human relaxin, administered as an intravaginal gel, on cervical ripening in pregnant women at term with an unfavourable cervix.

METHODS

Ethical approval for this study was obtained from the local ethics committees of the three participating centres.

Correspondence: Dr J. Brennand, Department of Obstetrics, Glasgow Royal Maternity Hospital, Rottenrow G4 0NA, UK.

Ninety-six women, comprising nulliparous and parous patients, were recruited to the study. All women had a singleton pregnancy of at least 37 weeks of gestation with a cephalic presentation and a modified Bishop score[10] of 4 or less. Women with a uterine scar, ruptured membranes or evidence of placental abruption or placenta praevia, were not recruited to the study. Other exclusion criteria were significant systemic disease, recent ingestion of aspirin or other nonsteroidal anti-inflammatory drugs, fetal malformation, growth retardation or macrosomia, and oligo- or polyhydramnios. The decision to induce labour was made by the woman's attending obstetrician and written informed consent was obtained before recruitment. The indication for induction in the majority of cases was either pregnancy induced hypertension or prolonged pregnancy.

The study involved a randomised, double-blind comparison of four treatment regimens: 0 (placebo), 1, 2 or 4 mg of recombinant human relaxin (rhRIx) in a gel vehicle. The randomisation sequence was generated in blocks of four by the Genentech Biostatistical Department and the code was stratified according to parity. A randomisation list accompanied each drug shipment. Primiparous women were assigned sequential numbers starting from the top of the list, and multiparous women were assigned sequential numbers starting from the bottom of the list. The study medication was supplied by Genentech, Inc. , as a two-part administration set: a stoppered 20 cm^3 glass vial of lyophilised rhRIx or placebo and a 5 cm^3 syringe of gel. The vial contained 0 mg or 12 mg rhRIx in isotonic citrate buffer. The syringe delivered 3 mL of sterile 4% methylcellulose gel in isotonic citrate buffer. The active doses were made by reconstituting 12 mg of lyophilised study medication in either 12, 6 or 3 mL of sterile water and combining this with the fixed 3 mL volume of methylcellulose gel.

Women were admitted to hospital in the afternoon before the day of induction. A medical and obstetric history was taken and a general examination performed. Fetal weight and amniotic fluid volume were estimated by ultrasound scan and a cardiotocograph performed. Blood was taken for haematology, coagulation profile, biochemistry, serum relaxin level and serum antibody to relaxin. Upon randomisation, the study medication was reconstituted using sterile water by one of the hospital pharmacy staff and delivered to the labour ward. The baseline modified Bishop score was recorded and the study gel administered intravaginally to the posterior fornix that evening. The woman remained recumbent for 1 hour following gel application. Blood pressure, pulse, respiration rate, uterine activity and fetal heart rate were monitored hourly for 4 hours post-treatment, and then every 4 hours for at least 24 hours or until delivery. Observations were suspended overnight if the woman was asleep. Blood was taken at 1, 4, 15 and 24 hours after gel administration for serum relaxin concentrations.

The following morning, at approximately 15 hours post-treatment, a vaginal examination was performed by the same investigator and the modified Bishop score recorded after which prostaglandin E_2 (PGE_2) gel 2 mg was instilled intravaginally. Further PGE_2 gel, 1 mg or 2 mg, was given after a 6-hour interval as required. Amniotomy was performed when the cervix was at least 3 cm dilated and fully effaced, and oxytocin administered according to hospital protocol. Uterine activity and fetal heart rate were monitored continuously throughout labour. If labour occurred before the planned 15 hour assessment, the modified Bishop score was recorded at the onset of labour and the woman managed as above. The onset of labour was defined as the onset of regular painful contractions with evidence of progressive cervical dilatation of the cervix. For women delivered by caesarean section before the second stage of labour, the length of the first stage was taken to be the total length of labour.

At delivery, umbilical cord blood was taken to assay relaxin concentration and blood gas analysis, and maternal blood for serum relaxin concentration. Apgar scores were recorded at 1 and 5 minutes post-delivery. Twenty four hours after delivery, maternal blood was collected for repeat haematology, coagulation profile, routine biochemistry and serum relaxin concentration. A general physical examination was also performed. Women were reviewed 6 weeks post-delivery when serum relaxin levels were measured and any postnatal problems documented.

The primary outcome measure was the change in modified Bishop score between baseline and 15 hours post-treatment. Secondary outcome measures included duration of first and second stage of labour, prevalence of 'spontaneous' labour and caesarean section, and oxytocin and total PGE_2 requirement. Maternal safety measures included vital signs, haematology and biochemistry screening. Fetal safety measures included stillbirth, neonatal death, fetal heart rate disturbances, Apgar scores, cord blood gases, need for resuscitation and incidence of neonatal morbidity.

Sample size and power consideration

Ninety-six women were recruited to the study with 24 women in each of the four treatment groups. The primary outcome measure, change in modified Bishop score, was first to be analysed by ANOVA to take account of any effect of dose influencing modified Bishop score. If a significant effect were found subsequent pairwise comparisons were to have been performed. The determination of sample size was based

Table 1. Subject characteristics at recruitment by treatment group. Age and gestation analysed by ANOVA; parity and baseline modified Bishop score analysed by Cochran Mantel-Haenszel test. Values are given as mean (SD).

Characteristic	Treatment group				*P*
	Placebo ($n = 23$)	1 mg ($n = 23$)	2 mg ($n = 25$)	4 mg ($n = 25$)	
Age (years)	27·0 (21·3–32·7)	26·8 (21·3–32·3)	26·7 (22·1–31·3)	25·8 (21·4–30·2)	0·796
Modified Bishop score	2·9 (2·0–3·8)	3·0 (2·3–3·7)	2·8 (1·9–3·7)	2·5 (1·4–3·6)	0·076
Gestation (weeks)	40·0 (38·5–41·5)	39·6 (38·2–41·0)	39·9 (38·8–41·0)	40·1 (38·9–41·3)	0·694
Parity					
0	18	20	22	19	
≥1	5	3	3	6	0·581

Table 2. Labour characteristics by treatment group. All parameters analysed by ANOVA. Values are given as mean (95% CI).

	Treatment group				*P*
	Placebo	1 mg	2 mg	4 mg	
Change in Bishop score	1·64 (1·0–2·3)	1·35 (0·7–2·0)	1·76 (0·5–3·0)	1·32 (0·8–1·9)	0·853
Duration of 1st stage (h)	4·9 (3·9–5·9)	5·0 (3·7–6·2)	5·3 (4·1–6·5)	6·5 (5·1–7·9)	0·222
Duration of 2nd stage (h)	0·7 (0·4–1·1)	1·3 (0·8–1·7)	1·0 (0·5–1·5)	1·1 (0·6–1·5)	0·387
Total duration of labour (h)	5·6 (4·6–6·6)	6·2 (4·7–7·8)	6·3 (4·9–7·7)	7·6 (5·9–9·2)	0·286
Treatment to 1st stage (h)	22·3 (19·2–25·3)	23·7 (21·0–26·3)	26·9 (22·3–31·6)	23·1 (20·2–25·9)	0·202
Treatment to delivery (h)	28·0 (24·8–31·2)	29·9 (26·7–33·2)	39·3 (26·4–52·2)	36·7 (23·8–49·6)	0·306

on such pairwise comparisons. Assuming that the standard deviation and the between-treatment difference with respect to the change in cervical score are equal, a two-sided *t* test with a significance level of 1·67% will have 84% power to detect such a difference.

Statistical analysis

All data were recorded on standardised case record forms and analysed at Genentech Inc in California. Analysis of variance tests were performed on all continuous variables, and Cochran Mantel-Haenszel tests employed for discrete variables.

RESULTS

Numbers of women in the four treatment groups were as follows: placebo: $n = 23$, 1 mg: $n = 23$, 2 mg: $n = 25$ and 4 mg: $n = 25$. There were no statistically significant differences in the characteristics of the women in the four groups (Table 1).

The mean changes in modified Bishop score were 1·64 for placebo, 1·35 for 1 mg relaxin, 1·76 for 2 mg relaxin and 1·32 for 4 mg relaxin. There was no statistically significant difference in the increase in modified Bishop score between the four groups (Table 2). In addition, there was no significant difference between the four groups when nulliparae and parous women were analysed separately (data not shown). The length of the first and second stages of labour was similar in all treatment groups. There were no differences in the time from treatment to first stage of labour or from treatment to delivery between the groups (Table 2). Excluding the patients delivered by caesarean section from the analysis did not alter the results for any of the above variables (data not shown).

The majority of women required induction 15 hours after study medication and only a few women laboured before this time (Table 3). The use of PGE_2 and oxytocin is also illustrated in Table 3. There was no statistical difference in requirement for either drug between groups, and the doses employed were similar in each treatment group. There was no significant difference in the mode of delivery between the groups with the majority of women achieving a spontaneous vaginal delivery. The overall caesarean section rate for the study was approximately 20% and the apparent increase in section rate in the 2 mg group did not reach statistical significance. Again, there was no statistical difference in the variables displayed in Table 3 when primigravid and parous women were analysed separately, or when women delivered by caesarean section were excluded from the analysis (data not shown).

Maternal and neonatal safety outcomes

There was no significant difference in pre- and post-treatment measurements of pulse and respirations between the four groups. There was a tendency to lower blood pressures in the treatment groups when compared

Table 3. Delivery characteristics, oxytocin and PGE_2 requirements, by treatment group. Total oxytocin and PGE_2 use analsyed by ANOVA; mode of labour and delivery analysed by Cochran Mantel-Haenszel test. Values are given as *n* and mean (95% CI).

	Treatment group				
	Placebo ($n = 23$)	1 mg ($n = 23$)	2 mg ($n = 25$)	4 mg ($n = 25$)	*P*
Spontaneous labour	*				
No	21	22	20	22	0·314
Yes	2	1	5	2	
Women requiring PGE_2	18	18	21	20*	0·928
Total PGE_2 dose (mg)	2·1 (1·5–2·6)	2·1 (1·5–2·6)	2·6 (2·0–3·1)	2·3 (1·8–2·8)	0·553
Women requiring oxytocin	10	13	12	13	0·916
Total oxytocin dose (U)	2·4 (0·4–4·4)	2·7 (1·2–4·1)	2·6 (0·8–4·5)	2·4 (1·1–3·7)	0·993
Mode of delivery:	*				
Vaginal	16	14	13	16	0·446
Operative vaginal	2	6	4	5	
Caesarean section	4	3	8	4	

*Data missing for one subject in this group.

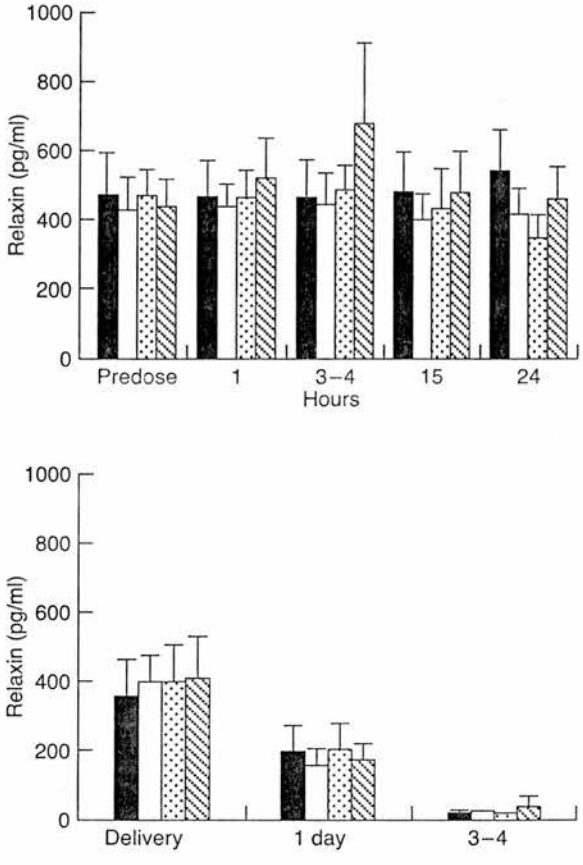

Fig. 1. Maternal serum relaxin concentrations (pg/mL): ■ = placebo, □ = 1 mg, ⊡ = 2 mg, ▨ = 4 mg, pre- and post treatment (mean, 95% CI).

with placebo and this was statistically significant for diastolic blood pressure: placebo −83 mmHg; 1, 2 and 4 mg rhRIx −77, −77 and 70 mmHg, respectively ($P \leq 0{\cdot}012$). This effect was seen over the first 14 hours following the start of treatment. Haematological and biochemical profiles were within the normal range for pregnancy. There was no association between treatment group and maternal outcome measures such as post-partum haemorrhage, infection and urinary retention.

There were significantly higher fetal heart rates in the treatment groups: placebo: 133 beats per minute (bpm); 1, 2 and 4 mg rhRIx: 136, 141 and 137 bpm, respectively ($P \leq 0{\cdot}011$). This effect was evident for approximately 24 hours post-treatment. There were no significant differences in 1 and 5 min Apgar scores, or cord blood gas results between the groups. Neonatal outcome measures such as meconium stained amniotic fluid, hypoglycaemia and hyperbilirubinaemia occurred with the same frequency in all treatment groups. There were no stillbirths or neonatal deaths in the study.

Relaxin concentrations

Maternal serum relaxin concentrations were assayed by Genentech. Concentrations following intravaginal administration of 1, 2 and 4 mg rhRIx were no different from the endogenous relaxin levels measured in the placebo group (Fig. 1). There was no correlation between baseline serum relaxin concentration and baseline modified Bishop score, or maximum serum relaxin concentration and change in modified Bishop score. Most cord blood relaxin concentrations were below the level of detection of the assay irrespective of treatment group. Details of the relaxin assay are 'in house' at Genentech.

DISCUSSION

A precise role for relaxin in the physiology of human parturition has yet to be defined. In a number of animals (eg. pig and rat), the onset of labour is clearly preceded by a surge in serum relaxin concentrations and this

appears to facilitate connective tissue remodelling in the reproductive tract. In human pregnancy serum relaxin concentrations peak at approximately 10 weeks of gestation and thereafter concentrations fall progressively towards term[11]. Labour itself is not associated with any further change in relaxin concentrations[11]. If relaxin plays a key role in cervical ripening it would be during the time of pre-labour that changes in serum relaxin concentrations might occur.

The corpus luteum is the primary source of relaxin during pregnancy, although extra-ovarian sites of production have been identified[12]. The fact that relaxin is not a pre-requisite for cervical dilatation has been demonstrated by the successful induction of labour in a woman with premature ovarian failure in whom serum relaxin levels were too low to record[13]. This finding also highlights the fact that relaxin production by other tissues is very small compared with ovarian production.

This study did not show any therapeutic effect of recombinant human relaxin on cervical ripening when administered as an intravaginal gel. There was no significant difference in mean change in modified Bishop score following treatment with 1, 2 or 4 mg of rhRIx compared with placebo. This is not surprising since pre- and post-treatment serum relaxin concentrations were the same in the placebo and active groups suggesting that relaxin was not absorbed from the preparation when administered via this route or that the dosages were too small. There was no association between endogenous serum relaxin levels and baseline modified Bishop score, or maximum serum relaxin concentrations and change in modified Bishop score. In addition, there was no change in endogenous levels throughout induction, labour and delivery which confirms previous findings. RhRIx appears to be safe and not associated with any clinically significant side effects. In view of the lack of evidence for absorption of rhRIx, which is reflected in the absence of clinical effect, the changes in maternal diastolic blood pressure and fetal heart rate, which were small and clinically irrelevant, may well have been spurious.

These findings are in keeping with a smaller study[14] employing recombinant human relaxin in a dose of 1·5 mg which also failed to demonstrate a significant effect on cervical ripening. Similarly, this dose of recombinant human relaxin was not associated with any adverse maternal or fetal complications. There could be a role for relaxin in cervical ripening without any parallel increase in circulating relaxin concentrations if it were acting at receptor level. However, to date, a receptor for relaxin in humans has not been identified.

The reduction in cervical collagen concentration necessary for cervical ripening can, in part, be explained by an increase in enzymatic collagen degradation. Labour is associated with an increase in circulating collagenase levels[15] and collagenase activity within cervical tissue. It has been reported that relaxin increases collagenase activity[16]. Relaxin receptors have been identified on human fibroblasts[17], which along with leucocytes are capable of collagenase production. A number of clinical trials have demonstrated a cervical ripening effect of porcine relaxin in pregnant women[6,7]. However, these trials were small and the women were of mixed parity. In addition, in one study[6], women in the treatment group tended towards higher baseline Bishop scores limiting the conclusions that can be drawn. Cervical ripening has been likened to an inflammatory response[18] and one potential mechanism whereby porcine relaxin, although purified, could stimulate cervical ripening is by provoking an immunological reaction resulting in neutrophil degranulation and release of collagenase, perhaps mediated by interleukin-8 which has recently been shown to be produced by the human cervix[19].

The effect of relaxin on myometrial contractility in different species is varied. An inhibitory effect of purified porcine relaxin on the spontaneous contractility of nonpregnant human myometrium has been demonstrated *in vitro*[20]. This effect has been confirmed on nonpregnant and pregnant myometrium from rats and pigs, but porcine relaxin had little, if any, effect on spontaneous or induced, pregnant or nonpregnant, human myometrial contractility *in vitro*[21]. More recently, human relaxin has been shown to have only a minor effect on human myometrial contractility in late pregnancy[22]. The data for uterine activity in this clinical trial were not assessed, but as there was no difference in duration of labour or need for augmentation between groups it is likely that contractility was unaffected.

Prostaglandins are currently the most successful agents for ripening the cervix pharmacologically[23] but they have the disadvantage of simultaneously stimulating uterine activity. Although theoretically relaxin meets the requirements for the ideal cervical ripening agent, that is ripening without contractility, this study has not demonstrated any cervical ripening effect of recombinant human relaxin. This may simply be due to incorrect choice of dosage. However, it seems more likely that the route of administration for this large polypeptide hormone was inappropriate. The intravenous route may be more suitable and deserves to be investigated before we can discard relaxin as a ripening agent.

Acknowledgements

We are grateful to Genentech, Inc. for their support in the coordination and financing of this trial. Dr M. M. Chou was awarded a Research Fellowship from the Taichung Veterans General Hospital.

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Received 24 June 1996
Accepted 8 January 1997

gyneco1711 P171 22-09-97 15:35:37

hb / A54171/OG7o027

Acta Obstet Gynecol Scand 1997; 76: 00–00
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Acta Obstetricia et Gynecologica Scandinavica
ISSN 0001-6349

ORIGINAL ARTICLE

The influence of amniotic fluid on prostaglandin synthesis and metabolism in human fetal membranes

JANET BRENNAND[1], ROSEMARY LEASK[2], RODNEY KELLY[3], IAN GREER[1] AND ANDREW CALDER[2]

From the Departments of Obstetrics and Gynaecology, [1]University of Glasgow and [2]University of Edinburgh, and the [3]MRC Unit, Centre for Reproductive Biology, Edinburgh

Acta Obstet Gynecol Scand 1997: 76: 00–00. © Acta Obstet Gynecol Scand 1997

Objective. To investigate the effect of amniotic fluid on prostaglandin synthesis and metabolism in the fetal membranes.
Design. A cell culture study of amnion and chorion obtained at elective cesarean section incubated with amniotic fluid collected following either spontaneous labor and delivery, or elective cesarean section.
Subjects. Fourty-eight pregnant women at 37–42 weeks gestation: 24 in spontaneous labor and 24 delivered by elective cesarean section.
Results. Significantly more PGE_2 and $PGF_{2\alpha}$ were produced by amnion and chorion treated with amniotic fluid from spontaneous labor compared with elective cesarean section. Spontaneous labor amniotic fluid favors PGE_2 and PGFM production by amnion and chorion respectively, while elective section fluid stimulates PGE_2 synthesis by both tissues (reflected as PGEM in chorion). Amniotic fluid, from either spontaneous labor or elective section, had no effect on the metabolism of exogenous PGE_2 or $PGF_{2\alpha}$ by chorion cells.
Conclusion. Spontaneous labor is associated with the presence of a substance in amniotic fluid which facilitates prostaglandin synthesis in the fetal membranes, but which is without effect on prostaglandin metabolism.

Key words: amniotic fluid; fetal membranes; labor; prostaglandin biosynthesis and metabolism

Submitted 30 January, 1997
Accepted 12 August, 1997

While the mechanisms underlying the process of parturition remain incompletely understood, prostaglandins appear to play an integral role. Supporting evidence includes the finding of increased concentrations of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ in amniotic fluid, and their corresponding metabolites in maternal plasma, in association with spontaneous labor (1, 2). In addition, prostaglandins are capable of inducing labor at all gestations (3) and are known to be potent stimulators of myometrial contractility. This view is, however, not adopted by all, and it has recently been suggested that the increase in prostaglandin concentrations in biological fluids is a result of events promoting labor, rather than in itself being a central mediator of the process (4).

The fetal membranes, comprising amnion, chorion and decidua, are a major source of the primary prostaglandins PGE_2 and $PGF_{2\alpha}$, and their inac-

Abbreviations.
COX I: cyclooxygenase I; DPBS: dulbecco's phosphate buffered saline; EGF: epidermal growth factor; IL-1β: interleukin-1β; PAF: platelet activating factor; PG: prostaglandin; PGDH: prostaglandin dehydrogenase; PGE_2: prostaglandin E_2; PGE_2M: 13, 14-dihydro-15-keto-PGE_2; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; PGFM: 13, 14-dihydro-15-keto-$PGF_{2\alpha}$; PGHS: prostaglandin endoperoxide synthase; TGFα: transforming growth factor α; TNFα: tumor necrosis factor α.

tive metabolites 13, 14-dihydro-15-keto-PGE_2 (PGEM) and 13, 14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) (5). The amnion, as a result of the action of prostaglandin endoperoxide synthase (PGHS), produces mainly PGE_2 (6, 7), but production of $PGF_{2\alpha}$ in lower concentrations has also been demonstrated (8). PGE_2 production by amnion is significantly increased in tissue obtained following spontaneous labor (6, 7, 9). Negligible amounts of prostaglandin metabolites are produced by the amnion reflecting the low level of activity of the main metabolic enzyme 15-hydroxy-prostaglandin dehydrogenase (PGDH) in this tissue (6, 7, 9). Chorion has the highest PGDH activity of the three tissues (6, 7) and under basal conditions PGEM is the major prostanoid of the chorion (9). In association with spontaneous labor there is a significant increase in the production of PGFM by this tissue (7). The decidua produces PGE_2 and $PGF_{2\alpha}$ in equal amounts (6) with a significant increase in $PGF_{2\alpha}$ production with the onset of labor (7).

Concentrations of both primary prostaglandins and their respective metabolites increase in association with spontaneous labor. However, the major increase in PGE_2 appears to occur prior to the onset of established labor, whereas $PGF_{2\alpha}$ increases during labor (10). This points to a role for PGE_2 in cervical ripening and the initiation of labor, while $PGF_{2\alpha}$ may have a role for the maintenance and progression of labor. This is supported by the finding that the increase in $PGF_{2\alpha}$ correlates directly with the duration of labor (10), and that amniotic fluid concentrations of $PGF_{2\alpha}$ are reduced in women with dysfunctional labor (11). Since amnion is principally involved in PGE_2 synthesis it has been extensively investigated as a potential regulator of prostaglandin dynamics in association with parturition.

Amnion is an avascular tissue which is in contact with both the maternal and fetal environment. Production of PGE_2 could therefore be regulated by a factor(s) present in amniotic fluid, the nature of which may be controlled by the fetus. Alternatively, the decidua, which is maternal in origin, or the chorion, may influence prostaglandin production by the amnion. Amniotic fluid contains stimulators and inhibitors of prostaglandin production. An endogenous inhibitor of prostaglandin synthase has been demonstrated in amniotic fluid (12), the activity of which decreases with advancing gestation and the onset of spontaneous labor. This suggests that the amnion is under tonic inhibition which is gradually lost as pregnancy progresses. Further evidence supporting the role of inhibitory activities includes the finding that conditioned media from amnion obtained at cesarean section will inhibit endometrial cell prostaglandin output (13), and that amniotic fluid contains compounds that appear to inhibit phospholipase activity (14). Stimulatory activities exist in amniotic fluid (15, 16) and there is an increase in this activity with advancing gestation. There is therefore a reciprocal change in the stimulatory and inhibitory properties of amniotic fluid that is gestation dependent (16). Examples of agents that stimulate amnion cell prostaglandin production include epidermal growth factor (EGF), transforming growth factor α (TGFα) and platelet activating factor (PAF), all of which are present in amniotic fluid in increasing concentrations during labor (17, 18, 19).

The chorion is an important site of prostaglandin metabolism, and any change in metabolic activity within this tissue could significantly alter active prostaglandin concentrations. Since amniotic fluid contains factors which influence prostaglandin production by amnion, it is possible that these agents could also alter the metabolic activity of the chorion. As no previous studies have assessed the effect of amniotic fluid on production and metabolism simultaneously, the purpose of this study was to determine the effect of amniotic fluid on prostaglandin production and metabolism by the fetal membranes, amnion and chorion.

Material and methods

Amniotic fluid was collected from two groups of pregnant women at term (37–42 weeks) with an uncomplicated pregnancy. The first group labored spontaneously and achieved a vaginal delivery without oxytocin augmentation ($n=24$); the second group was delivered by elective cesarean section for either breech presentation or previous cesarean section ($n=24$). Fetal membranes were collected from a third group of women undergoing uncomplicated elective cesarean section at term ($n=4$). Because it is known that there can be variation in the response of these tissues in culture each experiment was performed twice. Therefore, a total of four cell culture experiments is described, each one employing fetal membranes from a different subject. Amniotic fluid from different subjects was used in each of the four experiments.

Amniotic fluid collection and preparation

Amniotic fluid was collected at elective cesarean section by transmembranous amniocentesis and immediately with delivery of the baby following spontaneous labor under aseptic conditions. The amniotic fluid was centrifuged at 2000 rpm at room temperature for 20 minutes and the supernatant divided into aliquots for storage at −20°C

pending the cell cultures experiments. On thawing, the amniotic fluid was centrifuged again and then filtered through a sterile 0.20 μm mesh to remove any contaminating bacteria prior to addition to the culture system.

Cell culture preparation

The membranes were trimmed from the placenta immediately following delivery and transported in Dulbecco's phosphate buffered saline solution (DPBS) containing heparin (10 u/mL). The amnion and chorion were separated manually and any excess decidua was peeled from the chorion with fine tissue forceps. The amnion and chorion were rinsed thoroughly in DPBS and weighed. The tissues were soaked separately in DPBS containing gentamicin (Sigma, UK) 40 mg/500 mL and amphotericin B (Sigma, UK) 2.5 mg/500 mL for 1 hour. Following a further four washes with DPBS the amnion and chorion were shredded into 3–4 mm pieces using skin graft blades. The chorion was then pre-incubated at 37°C for 10 minutes with 50 mL of digestion medium (Gibco Culture Medium RPMI 16/40 with 2.5 g trypsin and 10 mg DNA-ase (both Sigma, UK) per 500 mL) to remove red blood cells. The amnion and chorion were incubated with digestion medium (approx. 5 mL/g amnion, and 5–7 mL/g chorion) in Erlenmeyer flasks with a stirring magnet at 37°C for 40 minutes. Each tissue was filtered through a 0.16 mm nylon mesh and the filtrate, containing the cells, collected in a sterile container. The remaining tissue was dispersed mechanically in complete culture medium with an adapted Eppendorf pipette and filtered through the 0.16 mm mesh. This mechanical dispersion and filtration was repeated twice. Complete culture medium consisted of RPMI 16/40 (Gibco, UK) with 25 mM Hepes Buffer plus L-Glutamine with the following additions: 50 mL/500 mL fetal calf serum (Gibco, UK); 5 mL/500 mL Penstrep solution (5000 iu penicillin/5000 μg streptomycin/ml, Gibco, UK); 5 mL of insulin-transferrin-sodium selenite media supplement (Sigma, UK). The culture medium was centrifuged at 1500 rpm for ten minutes and the supernatant decanted. This procedure was repeated three times and following the third spin the cells were resuspended in a fixed volume of complete culture medium for counting. The cells were counted and viability assessed using Trypan Blue exclusion. The cell preparations were then diluted to give approximately 2×10^5 cells/well. The amnion and chorion cells were plated out separately in standard 24 well plates.

All amnion and chorion cells were maintained in culture for 7 days, at which time the cells remained at confluence and there was no evidence of bacterial contamination.

Effect of amniotic fluid on amnion and chorion prostaglandin production

The purpose of this experiment was to investigate the effect of amniotic fluid on PGE_2 and $PGF_{2\alpha}$ production, and PGEM and PGFM production by amnion and chorion cell cultures respectively.

The plates of amnion and chorion prepared as described above were incubated in humidified 95% air; 5% CO_2 at 37°C for 24 hours. Amniotic fluid obtained at elective cesarean section ($n=6$) and following spontaneous labor ($n=6$) was added to the cells in volumes of 50, 100, 200 and 400 μL. Cells were also incubated in media alone, to act as control, and with phorbol myristoyl acetate 100 nM (Sigma, UK) to confirm that prostaglandin synthesis could be induced in these preparations. The total volume of each well was maintained at 1 mL. Amniotic fluid (100 μL) from each subject was also incubated alone for estimation of background primary prostaglandins and their metabolites. All experiments were performed in duplicate. The plates were incubated for a further 24 hours and then 0.5 mL aspirated from each well for prostaglandin measurement. The above experiment was repeated on a second set of fetal membranes using amniotic fluid from different subjects (again, $n=6$ for both groups).

Amniotic fluid and chorion cell metabolism of exogenous prostaglandins

The purpose of this experiment was to investigate the effect of amniotic fluid on the metabolism of exogenous PGE_2 and $PGF_{2\alpha}$ to their respective metabolites by chorion cell cultures.

A chorion cell culture was prepared as previously described and plated out at 2×10^5 cells/well. The plates were incubated for 24 hours in humidified 95% air; 5% CO_2 at 37°C. Amniotic fluid collected following elective cesarean section ($n=6$) and spontaneous labor and delivery ($n=6$) was added to the cells and the plates incubated for a further 24 hours. Chorion cells and 400 μL of each amniotic fluid sample were incubated alone to enable estimation of background levels of the primary prostaglandins and their metabolites. The following additions were made (final concentrations): 500 ng PGE_2 (Upjohn Ltd, UK) and 500 ng $PGF_{2\alpha}$ (Upjohn Ltd, UK). For the final 4 hour incubation the plates therefore comprised wells containing: chorion cells alone; cells+400 μL amniotic fluid+500 ng PGE_2; cells+400 μL amniotic fluid+500 ng $PGF_{2\alpha}$; cells+500 ng PGE_2 (control);

cells+500 ng $PGF_{2\alpha}$ (control); 400 μL amniotic fluid alone. The final incubation volume in all wells was 1 mL. All experiments were performed in duplicate. The incubation fluid (0.5 mL) was oximated and stored pending radioimmunoassay. The experiment was repeated on a second set of chorion with different amniotic fluids (again, $n=6$ for each group).

Radioimmunoassay

A competitive binding radioimmunoassay was used to determine the PGE_2, $PGF_{2\alpha}$, PGEM and PGFM content of the media. The technique has previously been described in detail (20, 21). At the time of the experiment 0.5 mL of culture medium was oximated with an equal volume of methyl oximating solution. These samples were mixed thoroughly, stored at room temperature for 24 hours and then at 5°C pending radioimmunoassay. In brief, up to 50 μL of the oximated culture medium was assayed. The labels employed are the iodinated (125I) methyloximes of PGE_2, $PGF_{2\alpha}$, PGEM and PGFM (Pro-Gly-Tyr conjugate in each case). Antisera to the respective labels were raised in the rabbit. The assay was left overnight prior to separation of the antigen-antibody complex from the free antigen by a second antibody magnetic separation procedure. The intra-assay coefficient of variation was 12.3% for PGE_2, 10.5% for $PGF_{2\alpha}$, 8.4% for PGEM and 7.3% for PGFM. The inter-assay coefficient of variation was 13.3%, 13.9%, 13.9% and 13.3% for PGE_2, $PGF_{2\alpha}$, PGEM and PGFM respectively.

Statistics

One and two factor analysis of variance (ANOVA) was used to analyze the data. Where the data were not normally distributed, log transformation was employed prior to ANOVA.

Results

The effect of amniotic fluid on prostaglandin production by amnion and chorion

Fig. 1 illustrates the stimulatory effect of amniotic fluid, in a dose-dependent manner, on prostaglandin E_2 and prostaglandin $F_{2\alpha}$ production by amnion. Amniotic fluid from spontaneous labor stimulated significantly greater production of both prostaglandins compared with elective cesarean section fluid: PGE_2, $p<0.001$; $PGF_{2\alpha}$, $p<0.05$. These findings were reproduced in a second culture (data not shown). Amniotic fluid from spontaneous labor produced significantly more PGEM from chorion, in a dose-dependent manner, than

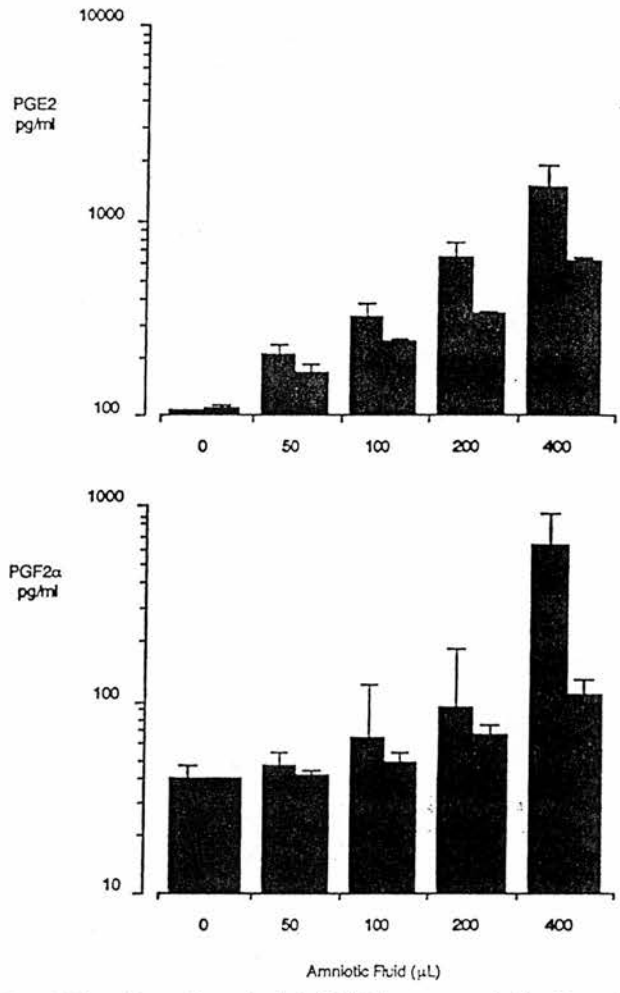

Fig. 1. The effect of amniotic fluid (AF) on postagladin E_2 and $F_{2\alpha}$ production by cultured amnion cells. ■ AF spontaneous labor ($n=6$); ▨ AF cesarean section ($n=6$). PGE_2, $p<0.001$; $PGF_{2\alpha}$, $p<0.05$. Results expressed as means (s.e.mean).

cesarean section amniotic fluid, $p<0.0001$ (Fig. 2). This was not confirmed in a second culture. Production of PGFM by chorion cells, reflecting $PGF_{2\alpha}$, was significantly greater in the presence of spontaneous labor amniotic fluid, compared with elective section fluid, at all doses, $p<0.003$ (Fig. 2). These findings were again reproduced in a second culture (data not shown).

The effect of amniotic fluid on metabolism of exogenous prostaglandins by chorion cells

Spontaneous labor and elective section amniotic fluid, in a dose of 400 μL, significantly stimulated PGEM and PGFM production compared with control, $p<0.05$ in chorion cell culture (Fig. 3) (reproduced in a second cell culture, data not shown). The production of both PGEM and PGFM was significantly greater in the presence of spontaneous

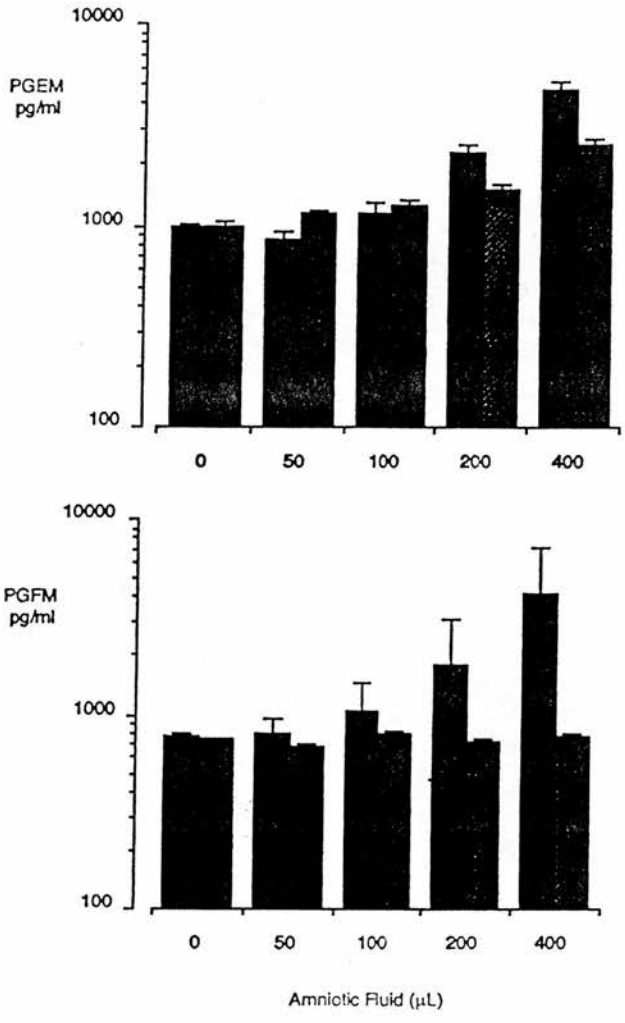

Fig. 2. The effect of amniotic fluid (AF) on postagladin metabolite production by cultured chorion cells. ■ AF spontaneous labor ($n=6$); ▨ AF cesarean section ($n=6$). PGEM, $p<0.0001$; PGFM, $p<0.003$. Results expressed as means (s.e.mean).

labor amniotic fluid compared with elective section fluid, $p<0.05$. In addition, the responsiveness of the chorion cells to phorbol myristoyl acetate, a stimulator of protein kinase C, is illustrated in Fig. 3. In both cultures, amniotic fluid from elective section stimulated significantly more PGEM production compared with PGFM, $p<0.0005$ (Fig. 4; data from one culture). Amniotic fluid from spontaneous labor stimulated significantly more PGFM production in comparison to PGEM, $p<0.05$ (Fig. 4), but was not statistically significant in the second culture ($p=0.056$; data not shown). The ratio of PGFM to PGEM production was 0.38 for chorion cells cultured with elective section amniotic fluid, compared with and FM:EM ratio of 4.6 when cultured in the presence of spontaneous labor amniotic fluid. This represents a 12-fold increase in FM:EM ratio.

There was no difference in PGEM production by chorion, following the addition of 500 ng PGE_2, when cultured in the presence of amniotic fluid from spontaneous labor or elective section compared with control (cells+500 ng PGE_2) in both cultures (Fig. 5). Similarly, there was no difference in the production of PGFM, following the addition of 500 ng $PGF_{2\alpha}$, by chorion cells cultured in the presence of amniotic fluid from elective section (both cultures) (Fig. 5). Amniotic fluid from spontaneous labor had no effect on PGFM production, following addition of $PGF_{2\alpha}$, in one culture, and stimulated significantly greater PGFM production compared with control (cells+500 ng $PGF_{2\alpha}$) in the second culture, $p<0.05$ (not shown).

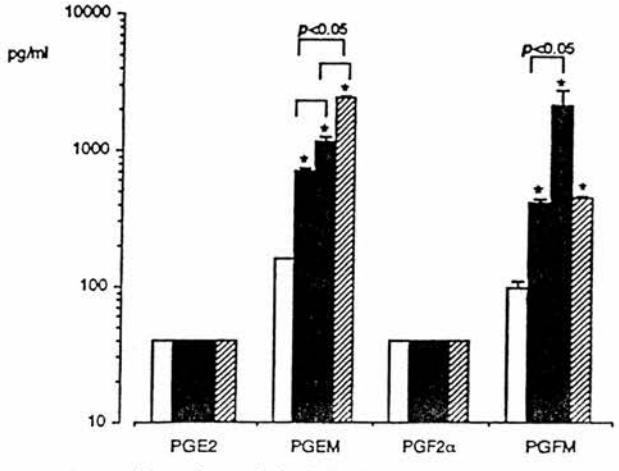

Fig. 3. The effect of amniotic fluid (AF) on postagladin/metabolite production by cultured chorion cells. □ control, ▨ AF cesarean section ($n=6$); ■ AF spontaneous labor ($n=6$). ?? phorbol myristoyl acetate. *=significantly different from control, $p<0.05$; ⌐¬ $p<0.05$. Results expressed as means (s.e.mean).

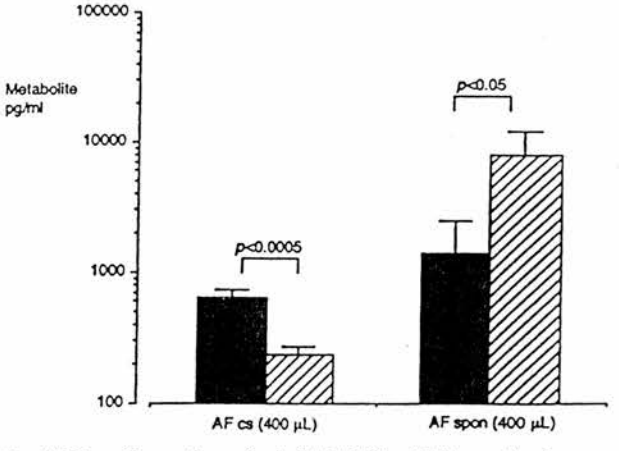

Fig. 4. The effect of amniotic fluid (400 µL) from elective section ($n=6$) and spontaneous labor ($n=6$) on PGEM compared with PGFM production by chorion cells. ▤ PGEM, ▨ PGFM. Results expressed as means (s.e.mean).

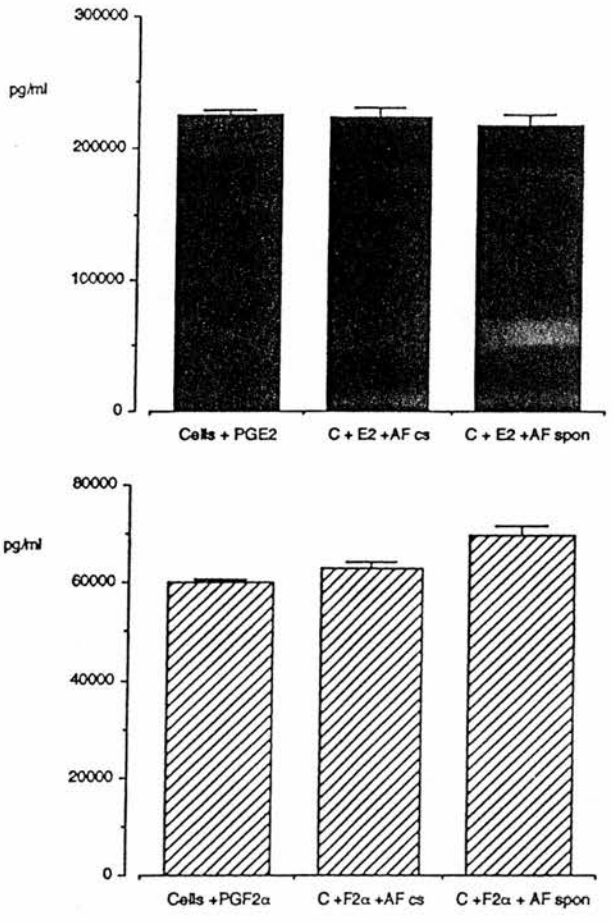

Fig. 5. The effect of spontaneous labor and elective amniotic fluid (both $n=6$) on the metabolism of exogenous PGE_2 and $PGF_{2\alpha}$. ▤ PGEM, ▨ PGFM. Results expressed as means (s.e.-mean).

Discussion

We have demonstrated that amniotic fluid from spontaneous labor and elective cesarean section at term is capable of stimulating prostaglandin production by amnion cell culture. This is in keeping with other workers who have shown increased PGE_2 production by amnion cells (15) and bovine seminal vesicles (16) incubated with amniotic fluid. In our experiments PGE_2 production was stimulated by amniotic fluid in a dose-dependent manner, confirming the findings of Mitchell et al. (22) who demonstrated PGE_2 stimulatory activity in amniotic fluid at all gestations. However, these workers did not find a difference in PGE_2 production in relation to parturition, unlike our experiments where amniotic fluid obtained at spontaneous labor had a significantly greater stimulatory effect than elective section fluid. Similarly, $PGF_{2\alpha}$ production was stimulated in these experiments and again responded to amniotic fluid in a dose-dependent manner. The response to spontaneous labor fluid was significantly greater than elective section fluid. It has previously been demonstrated that amniotic fluid will stimulate $PGF_{2\alpha}$ production by amnion, chorion and decidua (23) but in these experiments there was no difference between spontaneous labor and elective section fluid. Reddi et al. (24) revealed that amniotic fluid obtained late in labor significantly stimulated $PGF_{2\alpha}$ production by sheep seminal vesicle prostaglandin synthase, whereas fluid obtained before labor had no such effect. In our amnion cells cesarean section fluid had little effect on $PGF_{2\alpha}$ production, although a significant dose-response curve was achieved in one culture. In all cultures significantly more PGE_2 was produced compared with $PGF_{2\alpha}$ in response to both spontaneous labor and elective section fluid confirming that this is the major prostanoid synthesized by amnion (6, 7).

There was some variability in the response of the chorion cell cultures to amniotic fluid. PGEM production, reflecting PGE_2 synthesis, was stimulated by spontaneous labor amniotic fluid in a dose-dependent manner in one culture. Elective section amniotic fluid had a dose-related stimulatory effect on PGEM production in both cultures. Similarly, PGFM production, reflecting $PGF_{2\alpha}$ synthesis, was stimulated by amniotic fluid from the two groups in both cultures. There was a significant difference in the stimulatory effect of spontaneous labor amniotic fluid compared with that of elective section with regard to PGFM production. In the metabolism studies we confirmed the stimulatory effect of amniotic fluid (400 mL) from spontaneous labor and elective section on PGEM and PGFM production in both chorion cultures, although elective section fluid had no significant effect on PGFM production in one culture. Spontaneous labor amniotic fluid stimulated significantly more PGEM and PGFM production compared with elective section fluid in both cultures. In all chorion cultures elective section fluid stimulated significantly greater PGEM production than PGFM, whereas spontaneous labor fluid, although not significant in all cultures, tended to favor PGFM production.

We have shown, therefore, that amniotic fluid obtained at elective section and spontaneous labor is capable of stimulating prostaglandin production by both amnion and chorion and that there is a preference for which prostaglandin is produced: spontaneous labor favors PGE_2 and PGFM production by amnion and chorion respectively, while elective section fluid stimulates PGE_2 synthesis by both tissues (reflected as PGEM in chorion). These findings are in keeping with the fact that under basal conditions PGEM is the main product of the chorion (9), whereas $PGF_{2\alpha}$ is thought to be inte-

gral to the maintenance of established labor, with increasing concentrations of this prostaglandin as cervical dilatation advances (25, 26). There is a significant difference in the stimulatory activity of the amniotic fluid in relation to parturition, but we do not know from these experiments whether this is a result of gestationally related changes in inhibitory or stimulatory properties of the fluid. These findings are not dissimilar from those of Reddi et al. (24) who found, that as labor progressed, there was a simultaneous reduction in stimulatory activity and increase in inhibitory activity in amniotic fluid with respect to PGE_2 production, whilst the converse was true for $PGF_{2\alpha}$ production.

It has been postulated that changes in the metabolic capacity of the chorion may be just as important in the regulation of prostaglandin concentrations as any direct stimulatory effect on prostaglandin synthesis (27). In this study amniotic fluid from spontaneous labor or elective section had no effect on the concentration of PGEM or PGFM recovered from the chorion cell cultures, following the addition of PGE_2 and $PGF_{2\alpha}$ respectively, compared with control (cells+PG alone). These findings suggest that amniotic fluid has no significant effect on the metabolic pathway for prostaglandins in chorion, and in particular that there is no reduction in metabolism in relation to labor which could result in the increased prostaglandin concentrations seen at this time. This is in keeping with previous reports showing no change in prostaglandin metabolism in association with spontaneous labor (7, 28). This also accords with the finding that the localization of immunoreactive PGDH in chorion does not change with labor (29). In contrast, in cases of preterm labor associated with chorioamnionitis, it has been demonstrated that immunoreactive staining for PGDH is significantly reduced as a result of trophoblast destruction (30). This would enable prostaglandins to escape metabolism and thus facilitate myometrial contractility. In the present study, the metabolism by chorion of exogenous PGE_2 to its inactive metabolite PGEM was significantly greater than that of exogenous $PGF_{2\alpha}$ to PGFM ($p=0.0001$). The same was true in the presence of spontaneous labor amniotic fluid ($p<0.005$) and elective section fluid ($p=0.0001$). This is in keeping with the fact that the prostaglandin metabolizing enzymes are more able to inactivate PGE_2 compared with $PGF_{2\alpha}$ (31, 32).

We have confirmed that amniotic fluid can stimulate prostaglandin production by fetal membranes and demonstrated that it has no effect on prostaglandin metabolism in these tissues. What factor(s) present in amniotic fluid is responsible for the increased production has still to be clarified. It has been hypothesized that, since amniotic fluid at term is largely composed of fetal urine, the signal for the onset of parturition may come from the appropriately mature fetus (33). Fetal urine has been shown to stimulate PGE_2 production by amnion in a concentration dependent manner (34), and urine obtained from fetuses following spontaneous labor stimulates greater production of PGE_2 compared with fetuses delivered by elective cesarean section (35). Alternatively, recruitment of mediators such as interleukins and TNFα, resulting in an inflammatory process, may play a role in the onset of parturition, and prostaglandins will enhance the inflammatory role of such cytokines. Indeed, such a mechanism has been proposed for the changes associated with cervical ripening (36). An example of cytokine recruitment is the increased concentration of IL-1β which has been reported in women in preterm labor associated with infection (37) and in women in spontaneous labor at term (38), and this cytokine is known to be capable of stimulating PGE_2 production by amnion cell culture (39).

Although we have looked at prostaglandin synthesis and metabolism by fetal membranes in parallel, it must be remembered that the *in vitro* situation is not necessarily a true reflection of events occurring *in vivo*. The amnion and chorion were separated in these experiments and therefore any modulatory effect that these tissues may exert on each other will have been lost. Chorion-conditioned medium stimulates PGE_2 production by amnion (40) and chorion cells themselves are capable of interleukin production (41). Importantly, the membranes were devoid of decidua, which may influence these avascular tissues in a paracrine fashion. In particular, studies have shown that a decidual product is capable of inhibiting prostaglandin production by amnion (42) and that decidual cells contain an inhibitor of cyclooxygenase I (43).

In summary, our experiments confirm the stimulatory effect of amniotic fluid on prostaglandin production by amnion and chorion and demonstrate that this activity is greater in association with spontaneous labor. However, labor had no effect on the metabolizing capacity of the chorion suggesting that it is increased production rather than a reduction in prostaglandin metabolism which is responsible for the increased prostaglandin concentrations related to parturition.

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Address for correspondence:
Dr Janet Brennand
Department of Obstetrics and Gynaecology
The Queen Mother's Hospital
Yorkhill
Glasgow
G3 8SJ
UK

Prostaglandins, Leukotrienes and Essential Fatty Acids (1998) **58**(5), 369–375

Mechanisms involved in the stimulatory effect of amniotic fluid on prostaglandin production by human fetal membranes

J. E. Brennand,[1] R. Leask,[1] R. W. Kelly,[2] I. A. Greer,[3] A. A. Calder[1]

[1]Department of Obstetrics and Gynaecology, University of Edinburgh, Edinburgh, UK
[2]MRC Unit, Centre for Reproductive Biology, Edinburgh, UK
[3]Department of Obstetrics and Gynaecology, University of Glasgow, Glasgow, UK

Summary This study aims to investigate potential mechanisms involved in the stimulatory effect of amniotic fluid on prostaglandin production by fetal membranes. A cell culture study of amnion and chorion was obtained following elective caesarean section, incubated with amniotic fluid collected at term (37–42 weeks' gestation) following either spontaneous labour ($n = 6$) or elective caesarean section ($n = 6$). The effect of addition of cycloheximide and actinomycin D (inhibitors of translation and transcription respectively), and staurosporine and genistein (inhibitors of protein kinase C and tyrosine kinase respectively) to these cultures was investigated. ANOVA was employed for statistical analysis. Cycloheximide and staurosporine significantly inhibited the stimulatory effect of spontaneous labour and elective section amniotic fluid on PGE_2 production by amnion, and PGEM production by chorion. Genistein significantly inhibited the stimulatory effect of spontaneous labour amniotic fluid on PGE_2 and PGEM production by amnion and chorion respectively. The stimulatory effect of amniotic fluid on prostaglandin production is dependent on new protein synthesis, presumably cyclooxygenase (COX), and stimulation of cell signal transduction pathways involving protein kinase C and tyrosine kinase.

Introduction

Prostaglandins appear to play a major role in the initiation and/or maintenance of labour in women. Concentrations of prostaglandins and their metabolites are increased in maternal plasma, urine and amniotic fluid in association with spontaneous labour.[1,2] In addition, prostaglandins are capable of inducing labour at all gestations,[3] and chronic administration of cyclooxygenase (COX) inhibitors such as aspirin have been shown to result in prolongation of pregnancy.[4] The intrauterine tissues, comprising amnion, chorion and decidua, are an important site of prostaglandin synthesis and metabolism. Increased prostaglandin production by amnion has been demonstrated in association with labour,[5,6] whereas prostaglandin metabolism appears to be unaltered by this process.[6,7] However, the regulation of prostaglandin production within these tissues remains to be elucidated.

Amnion is an avascular tissue. Thus, the factors capable of regulating prostaglandin production within amnion must be present either in amniotic fluid, immediately adjacent chorion, or maternal decidua. Amniotic fluid at term is composed largely of fetal urine and this provides a potential mechanism for fetal control of prostaglandin production, as would a factor in fetal lung secretions, which might be linked to maturation. Alternatively, the interaction between maternally and fetally derived tissues at the chorio-decidual interface may enable signals that are maternal in origin to influence prostaglandin production in the fetal membranes in a paracrine manner.

It has been demonstrated that amniotic fluid contains an endogenous inhibitor of prostaglandin synthesis,[8] and a number of stimulatory activities.[9,10] There appears to be a reciprocal change in the activity of stimulatory and inhibitory factors, which is gestation-dependent, such that amniotic fluid obtained from women in spontaneous

Received 23 December 1997
Accepted 10 March 1997

Correspondence to: Janet Brennand, Department of Obstetrics, The Queen Mother's Hospital Yorkhill, Glasgow G3 8SJ, UK, Tel. + 44 141 201 0550, Fax. + 44 141 357 3610

labour at term will stimulate significantly greater prostaglandin production by amnion than amniotic fluid collected at elective caesarean section.

The amnion is the most extensively investigated of the intrauterine tissues with regard to the control of prostaglandin production. Since prostaglandins are not stored, but rather released immediately following synthesis in response to external stimuli, the major control of prostaglandin action lies with the regulation of its synthesis. The release of arachidonic acid, the obligatory precursor of prostaglandins of the 2-series, from membrane phospholipids, is thought to be a rate-limiting step in prostaglandin synthesis in the presence of COX. Protein kinase C (PKC) is a calcium- and lipid-dependent enzyme which has a crucial role in cell surface signal transduction, leading to the activation of many cellular functions. PKC activity has been identified in human amnion, and it has been suggested that PKC participates in the mobilization of arachidonic acid, and the activation of COX, thus facilitating prostaglandin synthesis.

Two isoenzymes of cyclooxygenase have been identified. Expression of the inducible form of the enzyme (COX-2) is increased in association with labour,[11] suggesting that increased COX-2 activity may be of principal importance in producing the increase in prostaglandin concentrations seen in association with spontaneous labour. The purpose of this study is to investigate potential mechanisms involved in the stimulatory effect of amniotic fluid on prostaglandin production by fetal membranes.

MATERIAL AND METHODS

Amniotic fluid was collected from two groups of pregnant women at term (37–42 weeks) with an uncomplicated pregnancy. The first group laboured spontaneously and achieved a vaginal delivery without oxytocin augmentation ($n = 6$); the second group was delivered by elective caesarean section for either breech presentation or previous caesarean section ($n = 6$). Fetal membranes were collected from a third group of women undergoing uncomplicated elective caesarean section at term ($n = 2$).

Amniotic fluid collection and preparation

Amniotic fluid was collected at elective caesarean section by transmembranous amniocentesis and immediately with delivery of the baby following spontaneous labour under aseptic conditions. The amniotic fluid was centrifuged at 2000 rpm at room temperature for 20 min and the supernatant divided into aliquots for storage at −20°C pending the cell culture experiments. On thawing, the amniotic fluid was centrifuged again, then filtered through a sterile 0.20 μm mesh prior to addition to the culture system.

Mechanisms of stimulation of prostaglandin production by amniotic fluid

Amnion and chorion cell cultures were prepared as previously described.[12] The amnion and chorion cells were plated out separately in 24 well plates at a density of 2×10^5 cells per well. All amnion and chorion cells were maintained in culture for 7 days, at which time the cells remained at confluence and there was no evidence of bacterial contamination.

The plates were incubated in humidified 95% air; 5% CO_2 at 37°C for 24 h. Amniotic fluid obtained at elective caesarean section ($n = 3$) and following spontaneous labour and delivery ($n = 3$) was used in the experiment. 400 μl of amniotic fluid was added to each well before addition of the following treatments (final concentrations): 1 μM and 10 μM staurosporine (inhibitor of PKC); 1 μM and 10 μM genistein (inhibitor of tyrosine kinase): 2 μg and 4 μg actinomycin D (inhibitor of transcription); 10 μg and 20 μg cycloheximide (inhibitor of translation). Cells were incubated in culture medium alone and in the presence of amniotic fluid to act as control. In addition, 400 μl of amniotic fluid was incubated alone for estimation of background primary prostaglandin and metabolite concentrations. The final incubation volume in all wells was 1 ml and all experiments were performed in duplicate. The plates were incubated for a further 24 h in humidified 95% air; 5% CO_2 at 37°C. The incubation medium was oximated and stored pending radioimmunoassay. The experiment was repeated on a second set of amnion and chorion cell cultures with different amniotic fluids (again, $n = 3$ for each group).

Radioimmunoassay

Radioimmunoassay was used to determine the PGE_2, $PGF_{2\alpha}$, PGEM and PGFM content of the media, and this has previously been described.[13,14] At the time of the experiment 0.5 ml of culture medium was added to an equal volume of methyl oxime solution, resulting in conversion of prostaglandins to their respective stable methyl oxime derivatives. These samples were mixed thoroughly, stored at room temperature for 24 h and then at 5°C pending radioimmunoassay. In brief, up to 50 μl of the oximated culture medium was assayed. The labels employed are the iodinated (^{125}I) methyloximes of PGE_2, $PGF_{2\alpha}$, PGEM and PGFM (Pro-Gly-Tyr conjugate in each case). Antisera to the respective labels were raised in the rabbit. The assay was left overnight prior to separation of the antigen–antibody complex from the free antigen by a second antibody magnetic separation procedure. The intra-assay coefficient of variation was 12.3% for PGE_2, 10.5% for $PGF_{2\alpha}$, 8.4% for PGEM and 7.3% for PGFM. The inter-assay coefficient of variation was 13.3%, 13.9%,

13.9% and 13.3% for PGE_2, $PGF_{2\alpha}$, PGEM and PGFM, respectively.

Statistical methods

One and two factor analysis of variance (ANOVA) were used to analyse the data. Where the data were not normally distributed, log transformation was employed prior to ANOVA. Background prostaglandin and metabolite concentrations in amniotic fluid were subtracted in all experiments.

RESULTS

Control

Spontaneous labour and elective section amniotic fluid significantly stimulated PGE_2 production by amnion in both cultures ($P<0.05$), and this effect was significantly greater in the presence of spontaneous labour fluid compared with elective section fluid ($P<0.05$) (Fig. 1). The same was true for PGEM production by chorion cells in both cultures ($P<0.05$). Spontaneous labour amniotic fluid significantly stimulated PGFM production by chorion in both cultures ($P<0.05$), but elective section amniotic fluid had no effect in either culture (data not shown).

Cycloheximide

Cycloheximide significantly inhibited baseline PGE_2 production by amnion ($P<0.05$) (Fig. 2). The stimulatory effect of amniotic fluid was also significantly inhibited but not abolished in the presence of cycloheximide, at both concentrations, compared with control ($P<0.05$).

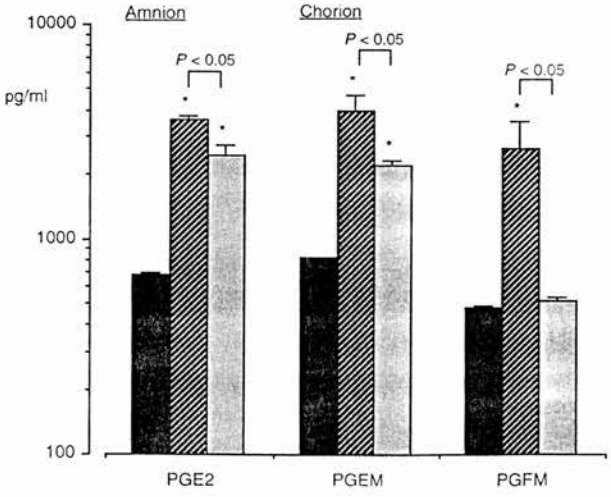

Fig. 1 The effect of amniotic fluid on PGE_2, PGEM and PGFM production by amnion and chorion cell cultures. (■) control, (▨) amniotic fluid (AF) spontaneous labour ($n = 3$), (□) AF caesarean section ($n = 3$). Significantly different from control, $P < 0.05$. Results expressed as means (SEM).

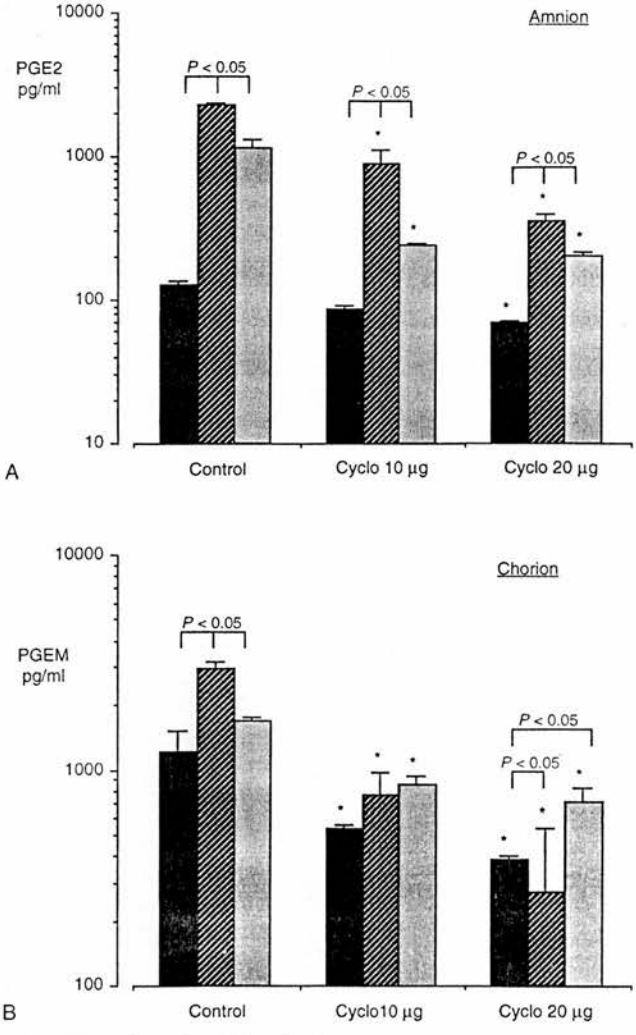

Fig. 2 The effect of cycloheximide on prostaglandin/metabolite production. (A) PGE_2 production by amnion cells. (B) PGEM production by chorion cells. (■) baseline, (▨) AF spontaneous labour ($n = 3$), (□) AF caesarean section ($n = 3$). * = significantly different from control, $P < 0.05$. Results expressed as means (SEM).

Similarly, cycloheximide 10 µg and 20 µg significantly inhibited baseline PGEM production by chorion, and significantly inhibited PGEM production previously stimulated by spontaneous labour and elective section amniotic fluid ($P<0.05$). Cycloheximide, at either concentration, had no effect on baseline or amniotic fluid stimulated PGFM production by chorion (data not shown).

Actinomycin D

Actinomycin D, 2 µg and 4 µg, significantly inhibited baseline PGE_2 production by amnion ($P<0.05$). The stimulatory effect of spontaneous labour amniotic fluid was significantly inhibited at doses of 2 µg and 4 µg, and that of elective section fluid was inhibited at a dose of 4 µg

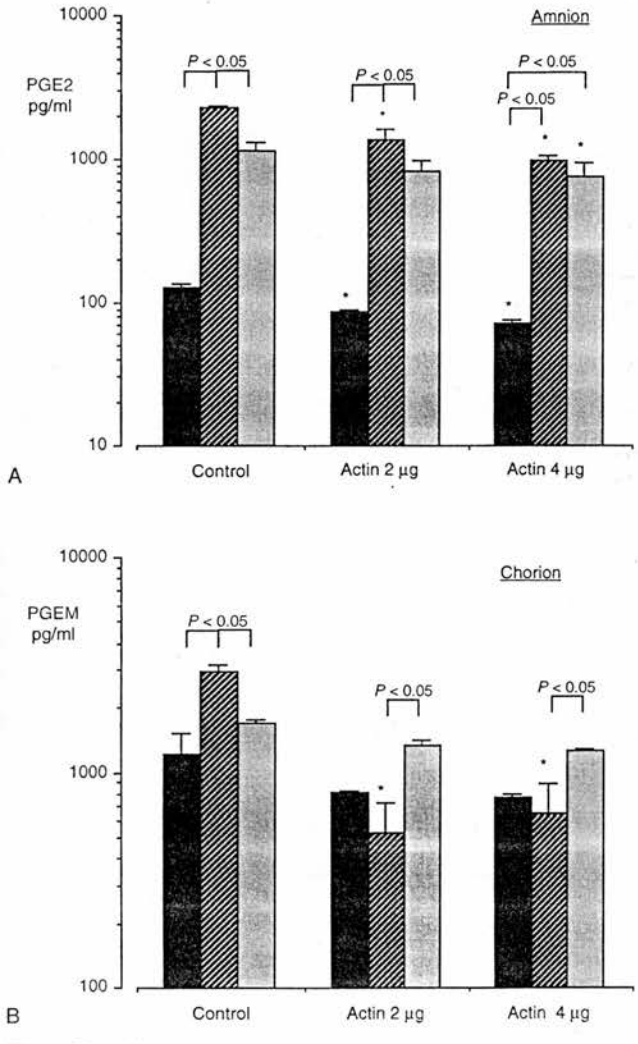

Fig. 3 The effect of actinomycin D on prostaglandin/metabolite production. (A) PGE_2 production by amnion cells. (B) PGEM production by chorion cells. (■) baseline, (▨) AF spontaneous labour ($n = 3$), (□) AF caesarean section ($n = 3$). * = significantly different from control, $P < 0.05$. Results expressed as means (SEM).

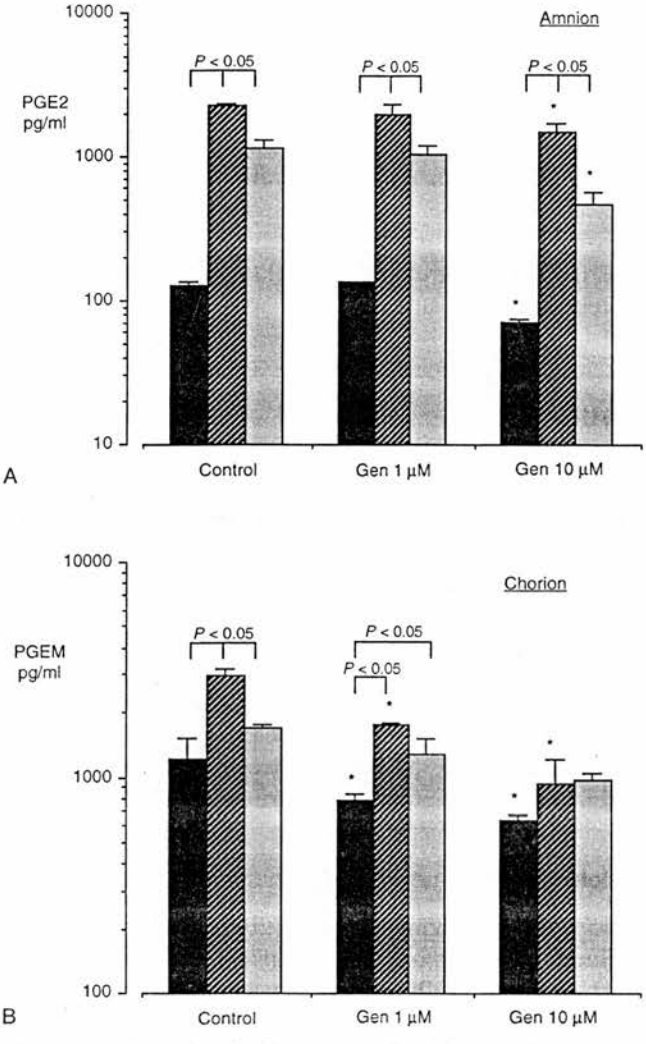

Fig. 4 The effect of genistein on prostaglandin/metabolite production. (A) PGE_2 production by amnion cells. (B) PGEM production by chorion cells. (■) baseline, (▨) AF spontaneous labour ($n = 3$), (□) AF caesarean section ($n = 3$). * = significantly different from control, $P < 0.05$. Results expressed as means (SEM).

($P < 0.05$). Actinomycin D had no effect on baseline PGEM production by chorion, but significantly inhibited the stimulatory effect of spontaneous labour amniotic fluid at doses of 2 μg and 4 μg ($P < 0.05$). However, there was a variability in the response between different cell cultures in that actinomycin D did not significantly inhibit the stimulatory effect of spontaneous labour amniotic fluid in the second cell culture. Actinomycin D 2 μg inhibited baseline PGFM production ($P < 0.05$), but otherwise had no effect on PGFM (data not shown).

Genistein

Genistein 10 μM significantly inhibited baseline PGE_2 production ($P < 0.05$) (Fig. 4). In addition, the stimulatory effect of amniotic fluid from both groups was significantly inhibited but not abolished by genistein 10 μM. Genistein inhibited baseline PGEM production ($P < 0.05$) and the stimulatory effect of spontaneous labour amniotic fluid was inhibited by genistein 1 μM and 10 μM ($P < 0.05$). Genistein had no effect on elective section amniotic fluid-stimulated PGEM production. Genistein had no effect on PGFM production by chorion (data not shown).

Staurosporine

Staurosporine 1 μM and 10 μM significantly inhibited baseline PGE_2 production ($P < 0.05$) (Fig. 5). The stimulatory effect of amniotic fluid from both groups was also inhibited in the presence of staurosporine ($P < 0.05$). At a dose of 10 μM staurosporine completely abolished the stimulatory effect of elective section amniotic fluid. This

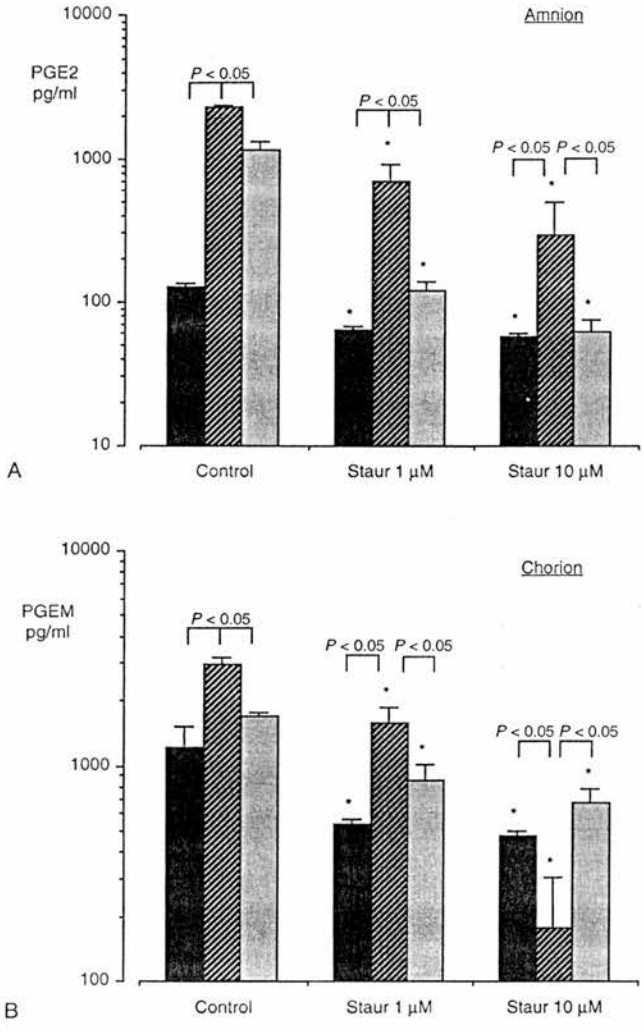

Fig. 5 The effect of staurosporine on prostaglandin/metabolite production. (A) PGE_2 production by amnion cells. (B) PGEM production by chorion cells. (■) baseline, (▨) AF spontaneous labour ($n = 3$), (▤) AF caesarean section ($n = 3$). * = significantly different from control, $P < 0.05$. Results expressed as means (SEM).

was not the case for spontaneous labour fluid which continued to stimulate greater PGE_2 production compared with cells treated with staurosporine alone. Staurosporine 1 μM and 10 μM significantly inhibited baseline PGEM production by chorion ($P<0.05$), and also inhibited the stimulatory effect of amniotic fluid from both spontaneous labour and elective section ($P<0.05$). Staurosporine had no effect on baseline PGFM production by chorion, but significantly inhibited the stimulatory effect of spontaneous labour amniotic fluid ($P<0.05$) (data not shown).

DISCUSSION

The results of these experiments demonstrate the stimulatory effect of amniotic fluid obtained at spontaneous labour and elective caesarean section on PGE_2 production by amnion, as previously reported.[6,7] Spontaneous labour amniotic fluid promoted significantly greater prostaglandin production compared with caesarean section fluid, suggesting the presence of either increased stimulatory activity or decreased inhibitory activity in association with labour. In addition, spontaneous labour amniotic fluid significantly stimulated EM and FM production in chorion, reflecting increased prostaglandin synthesis within this tissue, which is in keeping with our previous findings.[12]

Prostaglandin synthesis is regulated at a number of points. Protein kinase C can increase arachidonic acid availability directly by acting on phospholipase A_2 (PLA_2) or indirectly, via diacylglycerol at the level of the enzyme phospholipase C. In addition to its effect on substrate release, PKC is also able to stimulate transcription and translation, resulting in de novo synthesis of COX enzyme.[15] Stimulatory factors in amniotic fluid could therefore increase prostaglandin production by a direct effect on PKC, resulting in increased substrate release and COX synthesis. Alternatively, the site of action may be at the level of the phospholipases, again leading to increased substrate availability.

We employed staurosporine (a PKC inhibitor) to investigate a possible role for PKC in the increased prostaglandin production by amnion seen in response to amniotic fluid. Phospholipid-dependent PKC activity has been demonstrated in human amnion,[16] and activation of PKC has been associated with stimulation of prostaglandin synthesis in the initiation and maintenance of parturition.[17] The stimulatory effect of phorbol esters on prostaglandin production by amnion can be inhibited by a number of PKC inhibitors including staurosporine.[18] In our experiments, the addition of staurosporine to the amnion cultures resulted in significant inhibition of basal prostaglandin synthesis, and inhibition of amniotic fluid-stimulated prostaglandin synthesis, both spontaneous labour and elective section, suggesting that in amnion cultures PKC has a role in maintaining basal prostaglandin production, but is not the sole mechanism involved, since production was not abolished by staurosporine. Other investigators have shown that staurosporine had no significant effect on unstimulated prostaglandin production,[18,19] and may reflect differences in the duration of the cell cultures as the cell's ability to respond changes with time.[20]

The inhibition of amniotic fluid-stimulated prostaglandin synthesis by staurosporine suggests that PKC is involved in this process. However, spontaneous labour amniotic fluid continued to stimulate significantly greater prostaglandin production than caesarean section amniotic fluid, or basal output, suggesting an additional mechanism(s) in this process. The stimulatory effect of

caesarean section amniotic fluid was completely abolished by staurosporine 10 μM. This could be due either to the presence of lower concentrations of stimulatory factors in amniotic fluid prior to the onset of labour, or to the absence of stimulatory factors necessary to activate alternative or additional mechanisms of prostaglandin synthesis.

Cycloheximide significantly inhibited baseline prostaglandin production in our cultures, indicating that new protein synthesis is occurring in the amnion cultures at this time. This is in keeping with the findings of Zakar and Olson,[18] and confirms that the PGE_2 accumulating in the medium was produced de novo in a protein synthesis-dependent manner. Cycloheximide consistently inhibited the stimulatory effect of amniotic fluid, spontaneous and elective section, on prostaglandin production, indicating that new protein synthesis is necessary in this process.

Actinomycin D, an RNA synthesis inhibitor, significantly inhibited basal prostaglandin production by amnion suggesting that continuous RNA synthesis was contributing to prostaglandin output by these cell cultures at that time point in the culture system. This is in contrast to the findings of Zakar and Olson,[18] who found that unstimulated prostaglandin output was not affected by actinomycin D. Our findings with regard to the effect of actinomycin D on amniotic fluid-stimulated prostaglandin production were inconsistent between the two different cultures and may reflect culture variability. The effect of actinomycin D is known to be dose-dependent,[17,18] and at the doses employed in our experiments we would have expected inhibition if RNA synthesis was essential for the effect seen with amniotic fluid, and this reached significance in one culture. The finding that new protein synthesis, and perhaps mRNA production, are required for the stimulatory effect of amniotic fluid on prostaglandin production suggests that this effect is mediated by new enzyme synthesis, and it may be production of cyclooxygenase that is being inhibited by these agents. However, it cannot be assumed that cycloheximide and actinomycin D are inhibiting transcription and translation processes specific to amniotic fluid since these agents will inhibit all such mechanisms within the cell cultures.

Again, the finding that spontaneous labour amniotic fluid continued to stimulate significantly more prostaglandin production by cells treated with cycloheximide than caesarean section amniotic fluid suggests the presence of additional factors in spontaneous labour fluid which may be stimulating alternative pathways. Or, this may simply reflect an increased concentration of a single stimulatory factor in association with labour. Alternatively, caesarean section fluid may promote translation of existing mRNA leading to increased protein synthesis, presumably COX-2, whereas spontaneous labour amniotic fluid may stimulate both transcription and translation resulting in greater total protein production. Furthermore, fluid from spontaneous labour may have a greater effect on substrate availability (PLA_2/PKC) which would promote increased prostaglandin production without increasing the amount of COX in the cell.

The results of the experiments suggest that in amnion the stimulatory effect of amniotic fluid on prostaglandin production is mediated, at least in part, by protein kinase C activation and new protein synthesis. In our chorion cultures, the stimulatory effects of amniotic fluid, spontaneous labour and elective section, were also inhibited by staurosporine and cycloheximide, again implicating PKC activity and new protein synthesis. It is possible that a dual mechanism is operating, one increasing enzyme production and one increasing substrate availability, and PKC could be responsible for both.

In addition to PKC, tyrosine kinase has a pivotal role in controlling signalling within the cell, and hence influencing cellular metabolism, the cell cycle and growth. The receptors for a number of growth factors including insulin and the cytokine epidermal growth factor (EGF) have been shown to possess tyrosine kinase stimulatory activity.[21] We investigated a potential role for tyrosine kinase in the amniotic fluid-stimulated increase in prostaglandin production by employing the tyrosine kinase inhibitor genistein in our cell cultures. The effect of spontaneous labour amniotic fluid was consistently inhibited in both amnion and chorion cell cultures. This finding demonstrates that activation of tyrosine kinase, either directly or indirectly, is occurring in response to amniotic fluid, and confirms that signal transduction processes are important in mediating amniotic fluid's stimulatory effect.

We have not attempted to characterize regulatory substances in amniotic fluid and therefore can only speculate as to what these agents might be. There is considerable interest in the role of cytokines in the process of parturition, concentrations of which are increased in amniotic fluid of labouring women at term compared with non-labouring women.[22] It has been suggested that parturition is an inflammatory-mediated process, similar to the events taking place during cervical ripening. Cytokines could be involved in the regulatory process of prostaglandin production through PKC and tyrosine kinase stimulation, and stimulation of transcription and translation for essential enzymes such as cyclooxygenase.

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The Effects of Mifepristone on Cervical Ripening and Labor Induction in Primigravidae

CATHERINE L. ELLIOTT, MB ChB, JANET E. BRENNAND, MB ChB, AND ANDREW A. CALDER, MD

Objective: To compare the effects of 50 mg or 200 mg of oral mifepristone with placebo on cervical ripening and induction of labor in primigravid women at term with unfavorable cervices.

Methods: This was a double-blind study in which 80 primigravidae at term with a modified Bishop score of 4 or less were randomly assigned to one of three treatment groups. They were assessed at 24-hour intervals for 72 hours, after which labor was induced if it had not occurred spontaneously.

Results: Two hundred milligrams of mifepristone resulted in a favorable cervix (with a Bishop score greater than 6 or in spontaneous labor) in significantly more women than placebo (P = .01). An improvement in cervical ripening was seen in the group given 50 mg of mifepristone, but this was not statistically significant. There were more cesarean deliveries performed for fetal distress in the group treated with 200 mg of mifepristone than placebo, but this was not statistically significant and was not associated with any differences between groups in terms of neonatal outcome.

Conclusion: Mifepristone, a progesterone antagonist, is known to cause softening and dilation of the human early pregnant cervix and an increase in uterine activity. It is theoretically attractive for use as an adjunct in cervical priming and labor induction. In this study, 200 mg of mifepristone was significantly more likely to result in a favorable cervix than placebo. (Obstet Gynecol 1998;92: 804–9. © 1998 by The American College of Obstetricians and Gynecologists.)

Induction of labor involves promoting softening and dilation ("ripening") of the cervix and producing effective myometrial contractions. It has been shown that induction is more likely to have a successful outcome if the cervix can be ripened before the onset of contractions, whether spontaneous or augmented.[1] Prostaglandin E_2 (PGE_2) is now widely used to prepare the unfavorable cervix for parturition.[1–3] However, prostaglandin preparations may cause uterine hyperstimulation[2] or may fail to produce sufficient cervical ripening for labor induction to proceed.

Mifepristone (RU 486, Hoechst-Roussel, Uxbridge, UK) is a potent progesterone and glucocorticoid antagonist acting on the progesterone receptor.[4] Its main clinical application has been as an abortifacient; however, it has been noted that its administration causes marked cervical softening in the first trimester, either in conjunction with prostaglandins promoting medical abortion[5] or when used alone before vacuum aspiration of the uterus.[6,7] Mifepristone shows synergism with prostaglandins in causing termination of pregnancy in the first or second trimester.[5,8] It seems reasonable, therefore, to propose mifepristone as an agent for cervical ripening in the third trimester, particularly in conjunction with prostaglandins. In primates, mifepristone has been demonstrated to be efficacious, in combination with oxytocin, in achieving cervical dilation and induction of labor.[9] In humans, Frydman et al[10] have shown that administration of 200 mg of mifepristone on 2 consecutive days to women at term significantly increased the number entering labor and decreased the prostaglandin requirements of the remainder, as compared with placebo.

Our study evaluated two doses of mifepristone (50 mg and 200 mg) as compared with placebo for their effect on cervical ripening and subsequent induction of labor in primigravid women whose cervices were initially unfavorable for induction. We also evaluated the maternal, fetal, and neonatal safety of mifepristone when used for this purpose. Because mifepristone is also a potent glucocorticoid antagonist[4] and because it is known to cross the placenta,[11,12] we also assessed whether there was an increased risk of neonatal hypoglycemia after its antepartum use.

From the Department of Obstetrics and Gynaecology, Centre for Reproductive Biology, University of Edinburgh, Edinburgh, United Kingdom.

 0029-7844/98/$19.00
PII S0029-7844(98)00284-1

Materials and Methods

We conducted a placebo controlled double-blind trial in which two single oral doses (50 mg and 200 mg) of mifepristone were compared with placebo in a dose escalation study to assess their efficacy and safety for the induction of labor in primigravid women at term. In the first part of the study, the efficacy of a dose of 50 mg of mifepristone was compared with placebo. On the basis of an interim efficacy analysis of this dose compared with the placebo, we then determined whether a higher or a lower dose of mifepristone should be used in the second part of the study. Twenty-five women were included in each treatment arm in the first part. On the basis of the interim efficacy analysis, an increased dose of 200 mg was used in the second part of the study. In the second part of the study, 25 women received the higher dose of mifepristone and another five were randomly assigned to receive the placebo (the placebo groups from both parts of the study were pooled for the final analysis). The sample size was calculated to detect a 40% difference in the number of patients who went into spontaneous labor or who had a Bishop score greater than or equal to 6 comparing patients receiving mifepristone and patients receiving placebo with a power of 90% at the 5% significance level. Randomization was achieved by predetermined randomization code. Patients were allocated a number, and therefore treatment, in strict numeric order as they entered the study. The study was approved by the Lothian Research Ethics Reproductive Medicine Subcommittee.

The inclusion criteria for the study stated that patients were primiparous women aged 18 to 40 years with a normal, live, single, cephalic presentation. Gestation length was between 37 and 41 weeks 4 days as determined by a first-trimester ultrasound scan. Labor induction was scheduled 72 hours after treatment. Patients were offered the option of inclusion in the study if they had a modified Bishop score[13] of 4 or less. After written informed consent was obtained, neither the patient nor the physician had knowledge of whether a single oral dose of mifepristone or placebo was given. Patients were excluded if they showed signs or symptoms of the onset of labor, of placental insufficiency, or if they had any contraindication to the use of mifepristone. Before study medication was given, a modified Bishop score was assigned by vaginal examination. In most women, subsequent cervical assessments were performed by the same investigator, each of whom was blinded to the treatment allocations.

After entering the study, the women were examined as outpatients after 24 and 48 hours. Fetal wellbeing was assessed by the women keeping a 'kick-chart' for each 24-hour period and by a further cardiotocograph at each review visit. At the 24- and 48-hour reviews maternal blood pressure and pulse were recorded, the vaginal examination was repeated, and a Bishop score calculated.

If labor had not occurred within 72 hours after taking the allocated treatment the woman was admitted for induction. An initial dose of 1 mg of PGE_2 gel was inserted into the posterior fornix of the vagina unless the cervix was more than 3 cm dilated and fully effaced, in which case artificial rupture of the membranes was performed. The cervix was reassessed after 6 hours and (unless labor was established) a further dose of 1 or 2 mg of PGE_2 was given intravaginally. The examination was repeated every 4 hours, and when appropriate, artificial rupture of the membranes was performed. Oxytocin was administered as clinically indicated. The fetal heart rate was monitored continuously once labor was established.

A cord blood sample was obtained at delivery for blood gas analysis, biochemical and hematologic factors, and for assay of cortisol, ACTH, and mifepristone levels.

Neonatal blood glucose was monitored 1, 3, and 12 hours after delivery by means of a heel prick sample. Neonatal weight, pulse, and temperature were recorded after 24 and 48 hours, as were any adverse clinical findings in the mother or infant. A further sample of maternal blood was taken 24 hours after delivery. Both mother and baby were reviewed 1 week and 1 month after delivery; standard observations were recorded, and any adverse events were noted.

Data were stored on computer database, and analysis was performed using the Statistical Analysis Software (SAS) package (SAS Institute, Cary, North Carolina). Differences between the treatment groups were compared using the t test for continuous data and the χ^2 test for categoric data.

The primary efficacy measure was successful cervical ripening (ie, modified Bishop score of 6 or greater or the onset of spontaneous labor) within 72 hours of treatment administration.

Results

The patient characteristics are given in Table 1. One woman was 17 years old at inclusion into the study and one was at a gestation of 41 weeks and 5 days when she received treatment. Although these constituted violations of the protocol, both were included in the efficacy analysis. Proportional odds models were fitted to the data, with and without factors for maternal age, gestational age, weight gain, weight at booking, and pretreatment Bishop score. None of these factors were found to

1. Patient Characteristics

Characteristic	Placebo	Mifepristone 50 mg	Mifepristone 200 mg
mean ± SD	26.2 ± 5.9	25.8 ± 4.5	25.6 ± 3.3
n (wk + d), mean	40 + 6 (3.6)	40 + 5 (3.5)	40 + 6 (3.1)
)			
weight (kg),	62.2 ± 8.9	71.8 ± 13.6	69.8 ± 13.3
± SD			
rvical score, median	3 (1–4)	4 (2–4)	3 (1–4)
)			
pregnancy			
imester termination	2	1	4
iscarriage	4	5	1
	0	1	0

standard deviation

istically significant at the 5% level and were ed from the final model.

ll three treatment groups the most common for inclusion was prolonged pregnancy (longer) weeks). The next most common reason was ensive disorders.

main outcome measures are summarized in 2. Cervical ripening was deemed successful if neous labor had ensued or if the Bishop score eater than 6 before induction of labor 72 hours ne treatment administration. Cervical ripening ccessful for 64%, 48%, and 30% of the patients with 200 mg, 50 mg of mifepristone, and pla- espectively. This difference was statistically sig- for the 200 mg group ($P = .01$, odds ratio (OR) 5% confidence interval (CI) 1.34, 12.84). There statistically significant difference between the mifepristone group and the placebo group ($P =$ 2.36, 95% CI 0.66, 8.37). The number of patients ntaneous labor after 72 hours was 9 (36%), 8 nd 7 (23.33%) in the 200-mg, 50-mg and placebo respectively.

subsequent course of labor is summarized in and the modes of delivery in Figure 1. Similar

. Outcome at 72 Hours After Treatment

	Placebo ($n = 30$)	Mifepristone 50 mg ($n = 25$)	Mifepristone 200 mg ($n = 25$)
ous labor	7 (23.3)	8 (32)	9 (36)
with a BS	2 (6.7)	4 (16)	7 (28)
l cervical g*	9 (30)	12 (48)	16 (64)†

ishop score

e given as number (%).

ssful cervical ripening was defined as Bishop score greater being in spontaneous labor.

01 compared with placebo.

numbers of women in all three groups were given PGE_2, had an artificial rupture of membranes, and received oxytocin. The median amount of oxytocin required was 1095 mU, 5198 mU, and 5780 mU for the 200-mg, 50-mg, and placebo groups, respectively. This reduction in the requirement by the former group is not statistically significant. Significantly fewer women who received 50 mg of mifepristone had a cesarean delivery than those who were given placebo ($P = .033$, $\chi^2 = 4.55$); however, in the group given 200 mg of mifepristone there was no statistically significant difference from the placebo group in the overall number requiring cesarean delivery ($P = .075$). In the 200-mg treatment group, eight of nine cesareans were performed for fetal distress and the other for failure to progress. In the placebo-treated group, three of eight cesareans were for distress and five for failure to progress.

A wide range of maternal minor adverse events were reported in all treatment groups, most of which were complications of pregnancy, such as hemorrhoids or headache. There were no marked differences across the groups in the reporting of these. One patient in the 200-mg mifepristone treatment group had transiently increased liver function tests.

There were no episodes of antepartum fetal distress after recruitment to the study. In labor, fetal distress was considered severe if it required medical intervention (such as fetal blood sampling) or delivery. There were 12 (48%), 6 (24%), and 4 (13.3%) cases of fetal distress in the 200-mg mifepristone, 50-mg mifepristone, and placebo groups, respectively.

Blood was taken from the umbilical vein at delivery. Because of practical problems, this was not possible for all infants in the study. The blood gas levels, ACTH, and cortisol values from these samples are summarized in Table 4. There were no statistically significant differences between the groups in any of the cord gas levels. The analysis of ACTH and cortisol in the cord blood showed a wide range of values; the values for ACTH, in particular, show wide standard deviation. There was no significant difference between the 200-mg mifepristone group and the placebo group. The mean levels of mifepristone in the cord blood were .048 mg/L (standard deviation [SD] = .038) and .054 mg/L (SD = .047) after 50 mg and 200 mg of mifepristone, respectively. Figure 2 illustrates the relationship between the length of time to delivery after mifepristone administration and the cord blood levels of mifepristone. The Apgar scores of the infants were recorded at 1 minute and 5 minutes in all of the infants and at 10 minutes in eight infants. There were no significant differences in these scores across the treatment groups.

Hypoglycemia in the neonates was monitored after delivery by means of a heel-prick sample at 1, 3, and 12

Table 3. Course of Subsequent Labor

	Placebo (n = 30)	Mifepristone 50 mg (n = 25)	Mifepristone 200 mg (n = 25)
Time to onset of labor, median	81 h 15 min	80 h 20 min	75 h 50 min
Range	(17 h 40 min to 104 h 30 min)	(6 h 55 min to 100 h)	(9 h 45 min to 101 h 15 min)
Time to delivery, median	88 h 14 min	85 h 15 min	84 h 6 min
Range	(27 h 21 min to 113 h 35 min)	(15 h 12 min to 113 h 47 min)	(13 h 1 min to 110 h 49 min)
PGE_2 given	23 (76.7%)	17 (68%)	16 (64%)
Total median PGE_2 dose (mg)	3	3	3
Oxytocin administered	14 (46.7%)	8 (32%)	12 (48%)
Total oxytocin dose (mU), median (range)	5780 (210–19,650)	5198 (2060–20,800)	1095 (210–14,220)

PGE_2 = prostaglandin E_2.

hours after delivery. Hypoglycemia was defined as a reading of less than 2.2, which was the case in a total of 10, 11, and 10 infants of mothers who received 200 mg of mifepristone, 50 mg of mifepristone, and placebo, respectively. Only one infant in the study population was admitted to the Special Care Unit as a result of hypoglycemia; the mother of this infant had received placebo. Figure 3 illustrates the mean glucose levels in the neonates.

Neonatal jaundice was reported in seven (28%), two (8%), and two (6.7%) of the infants of mothers who received 200 mg of mifepristone, 50 mg of mifepristone, and placebo, respectively. All of these cases resolved spontaneously and were not considered clinically significant.

Discussion

Mifepristone was deemed to have successfully promoted cervical ripening in primigravid women at term with an initially unfavorable cervix if after 72 hours the woman was in spontaneous labor or the cervix had become favorable (Bishop score greater than 6). By these criteria, 200 mg of mifepristone had a significant effect on cervical ripening, whereas the effect of 50 mg was just below the level of statistical significance compared with the placebo. An improvement in the Bishop score confers an increased chance of successful labor induction[13] which may be of particular benefit to this group of women. These findings confirm those of Frydman et al[10] who gave 200 mg of mifepristone on 2 consecutive days to women at term. As the effect of 50 mg of mifepristone in our study just failed to reach significance levels, it seems likely that a slightly increased dose, such as 75 mg or 100 mg, may also significantly

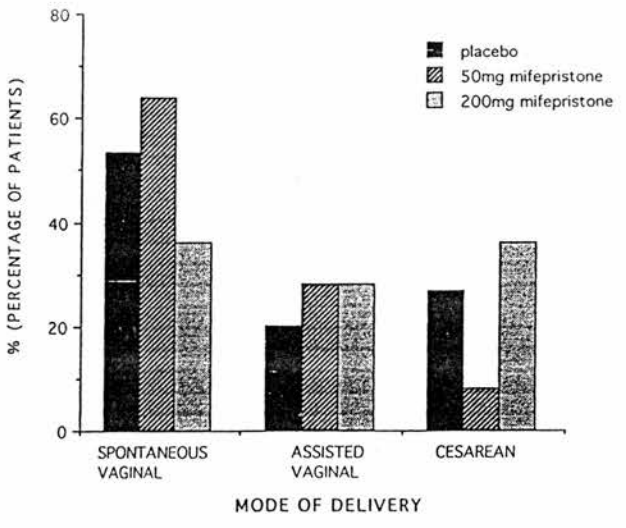

Figure 1. Mode of delivery after treatment with 200 mg, 50 mg of mifepristone, or placebo.

Table 4. Umbilical Vein Blood Parameters

	Placebo (n = 30)	Mifepristone 50 mg (n = 25)	Mifepristone 200 mg (n = 25)
po_2 (mmHg)	n = 6	n = 6	n = 12
Mean (SD)	32.3 (11.0)	30.3 (6.0)	38.9 (36.5)
Range	22.5–53.3	24.0–39.8	9.0–137.3
pco_2 (mmHg)	n = 6	n = 6	n = 12
Mean (SD)	39.5 (96)	40.9 (40)	41.4 (10.5)
Range	24.0–52.0	34.3–45.3	13.1–52.0
pH	n = 16	n = 21	n = 14
Mean (SD)	7.3 (0.1)	7.3 (0.1)	7.3 (0.1)
Range	6.9–7.4	6.9–7.4	7.1–7.4
Base excess (mmol/L)	n = 5	n = 6	n = 12
Mean (SD)	−4.7 (5.8)	−5.3 (2.1)	−7.7 (5.5)
Range	−14.6 ± −0.7	−7.9–−2.3	−22.2–−1.7
ACTH (mU/L)	n = 25	n = 21	n = 19
Mean (SD)	51.5 (91.5)	41.8 (43.9)	20.7 (22.6)
Range	3–433	3–208	3–84
Cortisol (mmol/L)	n = 24	n = 21	n = 19
Mean (SD)	484.5 (223.2)	498.6 (158.9)	522.1 (190.2)
Range.	234–1037	295–894	211–885

po_2 = partial pressure of oxygen; SD = standard deviation; pco_2 = partial pressure of carbon dioxide; ACTH = adrenocorticotropic hormone.

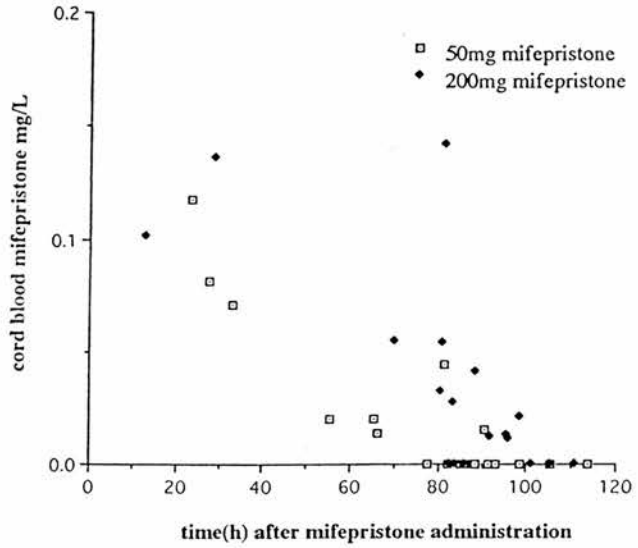

Figure 2. Mifepristone levels (ng/mL) in cord blood obtained at the time of delivery compared with the time to delivery after administration of 50 mg or 200 mg of mifepristone.

ripen the cervix and that doses greater than 200 mg could have a more pronounced effect.

As pregnancy progresses mifepristone becomes less efficacious at inducing abortion but its action in promoting cervical ripening is maintained.[4] This effect has been noted previously in Rhesus monkeys, in whom mifepristone alone induced cervical ripening but did not produce sufficient myometrial activity to affect delivery.[14] In combination with oxytocin, however, mifepristone was effective at inducing delivery in these monkeys.[9] As shown by this study, the most pronounced effect of mifepristone in the third trimester was in causing cervical ripening and increased sensitivity to oxytocin rather than labor induction. Women in this

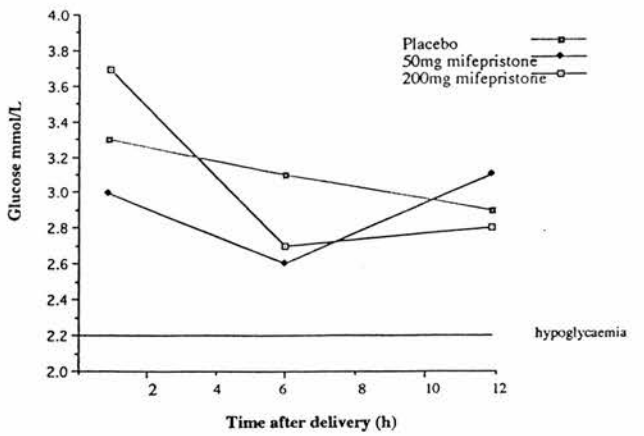

Figure 3. Mean neonatal blood glucose levels 1, 3, and 12 hours after delivery following antenatal administration of 50 mg or 200 mg of mifepristone.

study were initially given only 1 mg of PGE_2 after treatment administration because of concerns that increased uterine responsiveness and synergism between the two agents could lead to a risk of hyperstimulation. However, there were no episodes of hyperstimulation reported in any group.

The cervical changes after mifepristone administration have clinical implications. In addition, this finding may help to elucidate the processes whereby cervical ripening and the onset of labor occur spontaneously. The effect of antiprogesterones in causing cervical softening indirectly supports the role of progesterone in the maintenance of pregnancy.

In addition to efficacy, the other main outcome measures of the study were fetal and neonatal safety. Mifepristone has already been used extensively in women at higher doses than used here without major concerns regarding its safety. Our data showed no serious maternal side effects. Previous studies of placental transfer in the late second and third trimester of pregnancy in monkeys[11] showed that a steady-state equilibrium was relatively quickly established with a gradient between mother and fetus possibly limiting flux. It was also noted that the efficiency with which mifepristone crossed the placenta was significantly decreased in the third trimester compared with the second trimester of pregnancy. In the present study there was no evidence of altered glucocorticoid or mineralocorticoid action in the cord blood values obtained. As would be expected, the levels of mifepristone in the cord blood samples were higher in the group treated with 200 mg of mifepristone than in the group given 50 mg. The level of mifepristone in cord serum is inversely correlated with the length of time after administration that delivery occurs.

Fetal and neonatal wellbeing were assessed at each stage of the study. There was no antepartum evidence of fetal distress after administration of mifepristone, as assessed by cardiotocograph and by maternal record of fetal movement. Intrapartum distress was assessed clinically and was more often diagnosed in the women who received 200 mg of mifepristone than in women in the other two groups. In the 200-mg group, there were more cesarean deliveries for fetal distress and fewer for failure to progress, although there was no overall increase in the number of cesareans performed. In the group of women who received 50 mg of mifepristone there were fewer cesarean deliveries. No difference was found in cord pH values or Apgar scores between the three groups. Although this finding relates to small numbers of women, the possibility of increased fetal distress after treatment with 200 mg of mifepristone should be carefully evaluated in a larger trial before its use is recommended. In each group that received mife-

pristone, there was a decreased incidence of women requiring cesarean delivery as a result of failure to progress in labor as compared with placebo. In contrast to monkeys, labor induction after mifepristone administration in humans may result in a more efficient process, thus decreasing dystocia.

A dose of 200 mg of mifepristone had a significant effect on cervical ripening in this group of women, but it was associated with an increase in clinically suspected fetal distress, an effect not found with 50 mg of mifepristone. An intermediate dose may provide benefits in terms of cervical ripening without affecting fetal well-being. A larger trial is needed to ascertain more fully these effects.

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Address reprint requests to:
Catherine L. Elliott, MB ChB
Department of Obstetrics and Gynaecology
Central Middlesex Hospital, Action Lane
London, NW10 7NS
United Kingdom

Received December 29, 1997.
Received in revised form April 28, 1998.
Accepted May 7, 1998.